THE BIOCHEMICAL JOURNAL

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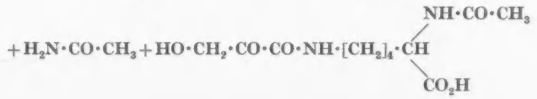
CORRIGENDA

Further observations on the oxidation of di-iodotyrosine derivatives

By Rosalind Pitt-Rivers and A. T. James

Volume 70 (1958)

p. 175, Fig. 1, last line of formulae *replace by*:



p. 176, col. 2, reference list

for **Sela, M. (1956).** ***read*** **Sela, M. & Sarid, S. (1956).**

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Physicochemical Properties of Hyaluronic Acid formed by *Streptococcus haemolyticus*

By B. S. BLUMBERG* and A. G. OGSTON
Department of Biochemistry, University of Oxford

and D. A. LOWTHER† and H. J. ROGERS
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 17 *January* 1958)

Examination of the sedimentation of a sample of protein-free hyaluronic acid, isolated from suspensions of *Streptococcus haemolyticus* by Lowther & Rogers (1956), suggested that it might have been partly degraded by hyaluronidase at some stage of its preparation. Accordingly the presence of hyaluronidase in the suspensions was looked for, and found. A preparation of hyaluronic acid was finally made under the same conditions, but with the addition of the hyaluronidase inhibitor 53 D_K (Rogers & Spensley, 1954, 1955); this proved to be much more highly polymerized than the first material.

The opportunity was taken of determining the specific refractive increment of protein-free hyaluronic acid.

EXPERIMENTAL

Culture of the organisms. A strain (T_{18}) of *Streptococcus haemolyticus* (Lancefield Group A) was grown in Hedley Wright broth (Wright, 1933), containing 1% (w/v) of glucose, 2·5% (w/v) of sodium β-glycerophosphate and 10% (v/v) of horse serum, and bacterial suspensions were prepared as described by Lowther & Rogers (1956). From two such cultures (*A* and *B*) suspensions of washed organisms were prepared, each containing 90 mg. of bacterial nitrogen, at a concentration of 0·5 mg. of bacterial N/ml. of suspending medium, and incubated at 35° for 2 hr. The fluid of the final suspension contained: 80 mM-sodium β-glycerophosphate; 25 mM-phosphate buffer, pH 7·0 (Sørensen); 20 mM-$MgSO_4$; 28 mM-glucose; 5 mM-glutamine. To one of the suspensions (*B*) was added 100 μg./ml. of the hyaluronidase inhibitor 53 D_K (Rogers & Spensley, 1954, 1955).

Separation of protein-free hyaluronic acid. After incubation the suspensions were cooled to 0° and the cells removed by centrifuging. The supernatant was dialysed under pressure for 4 days against flowing distilled water, and freeze-dried. These preparations were used without further treatment for physicochemical measurements. The preparation (*B*) which contained 53 D_K was further purified before chemical analysis: the hyaluronate was precipitated with cetylpyridinium chloride (Scott, 1955) and redissolved in 0·2 M-Na_2SO_4; the cetylpyridinium chloride was removed by treatment with fuller's earth; the hyaluronate was dialysed against water, de-ionized by passage through a short column of Zeo-Karb 225 (acid form) and the resulting hyaluronic acid was dried at 105°.

Analysis of hyaluronic acid. Glucosamine was determined by the method of Elson & Morgan (1933). Glucuronic acid in the sample *A* made without 53 D_K was determined by the method of Ogston & Stanier (1951); because of the small amount available, glucuronic acid in the sample *B* made with 53 D_K was determined by the colorimetric method of Dische (1947).

Estimation of concentration for physicochemical measurements. A representative sample of the freeze-dried preparation *A* was dissolved in water (3·24 mg./g. of solution); the material dissolved completely, to a clear solution. A weighed sample of this solution was dried to constant weight at 105°, giving 2·74 mg. dry wt./g. of solution; this showed that the preparation contained 84·5% of dry material. Subsequent solutions were made up by weight in buffer, with use of this value to obtain their contents of dry material. The value of the specific refractive increment obtained with *A* (see below) was used to determine the concentration of hyaluronic acid in *B*, by measurement of the area of its boundary diagram in the ultracentrifuge. Of the total refractive increment of *B*, 80% was due to hyaluronic acid and 20% to the accompanying 53 D_K.

Refractive increment. This was determined with the refractometer of Cecil & Ogston (1951) at 546 mμ.

Specific refractive increment. This was determined from the sedimentation diagram of a solution of *A*, which contained 0·344 g. of dry material/100 ml. of buffer. The area of the sedimentation boundary above the base-line (determined in a separate, parallel run with buffer alone) was determined by trapezoidal integration, corrected for dilution and converted into units of refractive index by use of the optical constants of the system (Baldwin, 1957).

Sedimentation. This was observed in a Svedberg oil-turbine ultracentrifuge at 900 rev./sec. and at 26–28°, by the method of Cecil & Ogston (1948).

Viscosity. The Couette viscometer of Ogston & Stanier (1953) was used to show that the solutions possessed no non-Newtonian viscosity. Subsequent measurements were therefore made with Ostwald viscometers of water-flow times 48, 108 and 200 sec.

Effects of streptococcal suspensions on hyaluronic acid. Suspensions of washed *S. haemolyticus* were incubated with

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solutions of (*a*) hyaluronic acid complex made from ox synovial fluid (Ogston & Stanier, 1950), (*b*) a protein-free preparation of hyaluronic acid from umbilical cord, and (*c*) streptococcal hyaluronic acid, in the normal suspending medium. Control experiments were performed with suspensions of streptococci which had been first heated to 70°. Estimations were also made of testicular hyaluronidase (Rhondase, Evans Medical Supplies Ltd.) in the presence of heated cells in the suspending medium. The suspensions were assayed for hyaluronidase activity both viscometrically (McLean & Hale, 1941) and by the turbidimetric method of Tolksdorf, McCready, McCullough & Schwenk (1949).

Buffers. Physicochemical measurements were made in 0·2 M-NaCl, 0·0077 M-Na_2HPO_4, 0·0023 M-KH_2PO_4, pH 7·3.

Table 1. *Glucosamine and glucuronic acid contents of hyaluronic acids from* Streptococcus haemolyticus

The values were obtained on material dried at 105° and have not been corrected for ash content.

Sample	Glucosamine (g./100 g.)	Glucuronic acid (g./100 g.)
A	36·1	42*
B	38·8	43†

* By decarboxylation (Ogston & Stanier, 1951).
† Colorimetric (Dische, 1947).

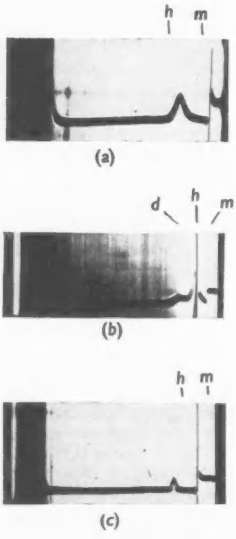

Fig. 1. Ultracentrifuge diagrams of hyaluronic acids from *S. haemolyticus*. Speed 900 rev./sec.; temperature 26–28°. *a*, Sample *A* 0·344 g./100 ml. of buffer 112 min. after reaching full speed; *b*, sample *B* 0·47 g./100 ml. 33 min. after full speed. The hyaluronic acid boundary is sharp compared with that of *A*. The small diffuse boundary due to the 53 D_K is seen ahead of the hyaluronic acid boundary, and all the light-absorbing material (546 mμ) is associated with this boundary; *c*, sample *B* 0·12 g./100 ml. 55 min. after full speed. The hyaluronic acid boundary is less sharp than in *b*, and the boundary of 53 D_K is not observable. Sedimentation is occurring from right to left. *m*, meniscus; *h*, boundary of hyaluronic acid; *d*, boundary of 53 D_K.

RESULTS

Composition. The percentages of glucosamine and of glucuronic acid for the two samples are given in Table 1.

Sedimentation. The results are given in Table 2 and illustrated in Figs. 1 and 2. Sample *A* gave

Table 2. *Measurements of sedimentation and viscosity made on hyaluronic acid from* Streptococcus haemolyticus, *and derived particle dimensions*

	Sample *A*	Sample *B*
$S^0_{20,w}$ (s)	2·5	6·5
$dS^{-1}_{20,w}/dc$ at $c=0$ [s^{-1} (g./100 ml.)$^{-1}$]	0·75	1·9
$[\eta]_e$ (g./100 ml.)$^{-1}$	2·3	6·0
10^{-3} (average particle wt.)	115	930
Hydrodynamic specific volume (ml./g.)	21	200
Axial ratio *a*/*b*	9	2

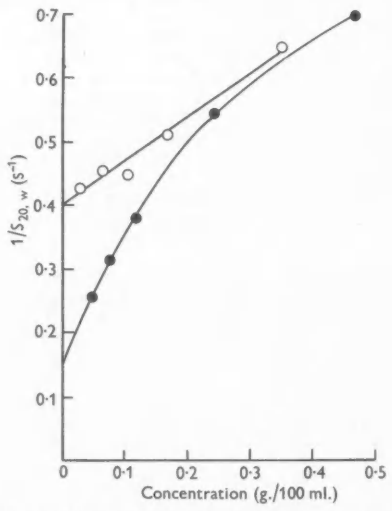

Fig. 2. Variation of $1/S_{20,w}$ (s^{-1}) against concentration, for samples of hyaluronic acid from *S. haemolyticus*. ○, Sample *A* formed in the absence of hyaluronidase inhibitor; ●, sample *B* formed in the presence of hyaluronidase inhibitor.

a diffuse boundary even at higher concentration, a low value of the sedimentation coefficient at zero concentration ($S^0_{20,w}$) and a low and linear rate of variation of sedimentation coefficient with concentration (dS^{-1}/dc); all these are typical of a relatively low degree of polymerization and probably of considerable polydispersity. The sharp boundary given by sample *B*, its high values of $S^0_{20,w}$ and of dS^{-1}/dc, and the variation of the last with concentration (Ogston & Woods, 1954) are all typical of a much higher degree of polymerization. At lower concentrations the boundary of *B* became diffuse, suggesting that it also is considerably polydisperse.

The pigmented material, 53 D_K, present in sample *B*, sedimented with a very diffuse boundary, well ahead of the hyaluronic acid (Fig. 1).

Viscosity. The intrinsic viscosities $[\eta]_c$ were obtained by linear extrapolation of $(\eta_{rel.}-1)/c$ to zero concentration. The values are given in Table 2.

Particle dimensions. These were calculated, approximately, by the method of Ogston (1953) from the values of S^0, dS^{-1}/dc and $[\eta]_c$. The results, given in Table 2, confirm that *B* is much more highly polymerized than is *A*.

Specific refractive increment. The value obtained for sample *A* was $1{\cdot}44 \pm 0{\cdot}01 \times 10^{-3}$.

Action of streptococci on hyaluronic acids. The properties of the hyaluronic acid produced in the absence of inhibitor were consistent with a partial breakdown of the mucopolysaccharide having occurred during preparation. Accordingly the presence of hyaluronidase was sought in washed suspensions of the organisms. Incubation at 37° of 1·5 ml. of a solution of hyaluronic acid complex (Ogston & Stanier, 1950) with cells equivalent to 1·0 mg. of bacterial nitrogen reduced the relative viscosity of the solution from 3·4 to 2·4 in 2 hr. This decrease would correspond with the presence of 0·16 Viscosity Reducing Unit (McClean & Hale, 1941) of hyaluronidase, and did not occur if the cell suspension had first been heated to 70°. Testicular hyaluronidase (Rhondase), diluted in suspending medium containing heated streptococcal cells, when incubated with hyaluronate solutions showed only about 6 % of the activity of dilutions made in buffer containing gelatin and pyrophosphate (Rogers & Spensley, 1954). It appears probable therefore that considerably more hyaluronidase was in fact present in the streptococcal suspensions than had been detected, the enzyme not being fully active under the conditions in which the hyaluronic acid was produced. When isolated streptococcal hyaluronate was incubated with 0·16 Viscosity Reducing Unit of testicular hyaluronidase for 2 hr. at 37° there was no decrease in the turbidity given by adding acidified serum under the conditions of Tolksdorf *et al.* (1949).

DISCUSSION

The hyaluronic acid formed in a washed streptococcal suspension under the conditions used by Lowther & Rogers (1956) is shown to have undergone degradation by hyaluronidase present in, or secreted by, the cells. Faber & Rosendal (1954) and MacLennan (1956*a*, *b*) have shown that many strains of streptococci which produce hyaluronic acid also produce hyaluronidase. The presence of the compound 53 D_K which inhibits both the action and liberation of hyaluronidase leads to the formation of a much more highly polymerized product; however, since it is difficult to prove that the enzyme was completely inhibited, the cells may be capable of forming still more highly polymerized hyaluronic acid.

The value of $1{\cdot}44 \times 10^{-3}$ obtained for the specific refractive increment of protein-free hyaluronate at 546 mμ agrees well with the value obtained less directly by Ogston & Stanier (1950). It is of similar magnitude to values reported for other saccharides, for example: dextran $1{\cdot}51 \times 10^{-3}$ at 589 mμ (Snyder, Isbell, Dryden & Holt, 1954), $1{\cdot}49 \times 10^{-3}$ at 546 mμ (Ogston & Woods, 1954); fructose $1{\cdot}41 \times 10^{-3}$ at 589 mμ (Jackson & Mathews, 1932). However, it disagrees markedly with the values obtained by Blumberg & Oster (1954) ($1{\cdot}8 \times 10^{-3}$ at 546 mμ) and by Laurent & Gergely (1955) ($1{\cdot}7 \times 10^{-3}$ at 436 mμ). These differences should be well outside the experimental error of the methods used. A low value would be obtained if the material were not completely dry; but to explain the discrepancies our material would have to contain 15 % of water even after drying to constant weight at 105°.

SUMMARY

1. The physicochemical properties of hyaluronic acid formed by a group A strain of *Streptococcus haemolyticus* in the presence and absence of the hyaluronidase inhibitor 53 D_K have been measured. The material formed in the presence of the inhibitor is much more highly polymerized.

2. Washed cells of *S. haemolyticus* have been shown to exert a hyaluronidase action; this is abolished by heating the cells to 70°.

3. A measurement has been made of the specific refractive increment of protein-free hyaluronic acid. The value obtained, $1{\cdot}44 \times 10^{-3}$, is believed to be accurate.

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Initial Stages in the Biosynthesis of Porphyrins

1. THE FORMATION OF δ-AMINOLAEVULIC ACID BY PARTICLES OBTAINED FROM CHICKEN ERYTHROCYTES

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(*Received* 28 *January* 1958)

The biosynthesis of porphyrins is thought to occur by the initial condensation of glycine with an unsymmetrical derivative of succinic acid (for review see Shemin, 1955) to form α-amino-β-oxoadipate, the latter being decarboxylated to δ-aminolaevulic acid, which is then converted through porphobilinogen into porphyrins. Observations indicating the formation of δ-aminolaevulic acid as an intermediate have been made with animal tissues (Shemin & Russell, 1953; Shemin, Russell & Abramsky, 1955; Neuberger & Scott, 1953; Berlin, Neuberger & Scott, 1956*a*, *b*; Dresel & Falk, 1953, 1956*a*, *b*) and in micro-organisms by Lascelles (1956, 1957) and Pawelkiewicz & Zodrow (1956). The evidence provided by these investigations for the formation of δ-aminolaevulic acid is quite strong, but somewhat indirect, being based largely on results obtained from isotope-dilution experiments, on a demonstration that this acid was much more active *in vitro* than glycine as a precursor of haem, and on the fact that δ-aminolaevulic acid is converted into porphobilinogen or porphyrins in a wide variety of systems (Gibson, Neuberger & Scott, 1955; Granick, 1954). A net synthesis of δ-aminolaevulic acid has not yet been demonstrated in systems *in vitro* and little evidence as to the nature of the enzymic mechanisms involved has been obtained.

The difficulty of demonstrating a net synthesis of the acid *in vitro* is mainly due to two causes. In the first place all the animal tissues used so far in studies in biogenesis of porphyrins, such as mammalian reticulocytes, red cells from non-anaemic birds, liver and bone marrow, produce on incubation *in vitro* so little δ-aminolaevulic acid that it cannot easily be measured by available methods without the use of isotopes. Secondly, the tissues mentioned metabolize the acid fairly rapidly, so that it does not accumulate to any appreciable extent. Both these difficulties have been overcome in the present work. It was found that erythrocytes obtained from anaemic chickens synthesize δ-aminolaevulic acid from glycine and succinate or α-oxoglutarate at a fairly high rate. Moreover, a particulate fraction was obtained from the lysates of such cells which retains the capacity to synthesize δ-aminolaevulic acid if suitably fortified with cofactors, but is free of enzymes concerned with the further metabolism of this acid. Chemical work on α-amino-β-oxoadipic acid (W. G. Laver, A. Neuberger & J. J. Scott, unpublished work) showed that this material loses carbon dioxide rapidly under physiological conditions, and in the present work we have therefore concentrated on demonstrating the formation of δ-aminolaevulic acid. For a preliminary account see Laver & Neuberger (1957).

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EXPERIMENTAL

Materials

δ-Aminolaevulic acid (ALA) was prepared by the acid hydrolysis of ethyl α-amino-β-oxoadipate (Neuberger, Scott & Shuster, 1956) and recrystallized from dry methanol–ether. [*carboxy*-^{14}C]Glycine, [α-^{14}C]glycine and [*carboxy*-^{14}C]succinic acid were supplied by the Radiochemical Centre, Amersham, Bucks. Coenzyme A (70–75% pure), adenosine triphosphate (ATP), and triophosphopyridine nucleotide (TPN) were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., pyridoxal 5-phosphate from Roche Products Ltd., Welwyn Garden City, Herts., and diphosphopyridine nucleotide (DPN, 75% pure) from Boehringer und Sochne, Mannheim, Germany. Samples of L-penicillamine and D-penicillamine were kindly supplied by Dr S. Wilkinson of the Wellcome Chemical Laboratories, Beckenham, Kent. All other chemicals were commercial samples of A.R. grade used without further purification.

Methods

Production of anaemia and preparation of haemolysates. Some difficulty was experienced in producing a degree of anaemia required for high activity of the final preparations without losing too many birds. Phenylhydrazine hydrochloride (generally 40 mg./kg. body wt.) gave a mortality rate of about 50%. An equivalent dose of acetylphenylhydrazine gave a mortality rate of about 20%, but the degree of anaemia produced was not sufficiently severe. A combination of phenylhydrazine and of its acetyl compound was found to be most satisfactory, active preparations resulting with a mortality rate of about 10%. A mixture (2·5 ml./kg. body wt.) of equal volumes of aqueous 2% (w/v) acetylphenylhydrazine and 2% phenylhydrazine hydrochloride, neutralized with NaOH, was injected intramuscularly into white Leghorn laying hens weighing 2–3 kg. After a period of 63–64 hr., veterinary Nembutal (Abbott Laboratories, London) (30 mg./kg. body wt.) and heparin ($1–5 \times 10^3$ units/kg.) were injected into the wing vein, the neck feathers were removed and the blood was collected into a chilled container by cutting the external jugular vein. The red cells were separated by centrifuging at 2° and washed twice with cold 0·9% NaCl, care being taken not to remove any of the light-coloured upper layer which appeared to consist largely of immature erythrocytes. The packed, washed red cells were then thoroughly chilled in ice and lysed by the addition with thorough stirring of 2 vol. of water also cooled to 0°. After 4 min. at 0° isotonicity was restored to the haemolysates by the addition of solid KCl (1·15 g./100 ml. of added water) to give a completely fluid preparation free of clots or gels of any kind.

Fractionation of the haemolysate and preparation of particles. The haemolysate was centrifuged at 3500 *g* at 0° for 30 min.; the supernatant fluid was removed and discarded unless it is otherwise stated. The residue was composed of two distinct layers, both about equal in volume. The light-coloured loosely-packed upper layer of the residue was carefully removed with a pipette and the particles were washed by centrifuging (3500 *g* for 10 min.) with cold 1·15% (w/v) KCl solution containing 2 mM-KH_2PO_4–K_2HPO_4 (pH 7·4), until the washings were free of haemoglobin (about six washings). Unless it is otherwise stated the tightly packed, dense, dark-coloured lower layer of the residue was discarded. The resulting preparation of particles was usually diluted with an equal volume of 1·15% KCl to facilitate pipetting, and any small lumps or clots which may have formed (due to contamination by the lower layer of the residue) were removed by straining through muslin. The washed particles were stored at 0° until use, but often lost their activity if kept for more than a few hours. Freezing and thawing reduced by half their capacity to form ALA from glycine and α-oxoglutarate, and completely destroyed the activity when α-oxoglutarate was replaced by succinate. In ten preparations the average volume of washed particles, packed at 3500 *g* for 10 min. in 1·15% KCl, obtained from 100 ml. of packed red cells was 65 ml.

Incubation. Samples were incubated in unstoppered conical flasks or wide-bore test tubes with shaking at about 100 shakes/min. at 38°. Anaerobic incubations were done *in vacuo* in Thunberg tubes without shaking.

Isolation of protoporphyrin. Protoporphyrin methyl ester was prepared from haemoglobin by the method of Grinstein (1947) and plated for counting as the crystalline copper complex (Dresel & Falk, 1954).

Isolation of radioactive δ-aminolaevulic acid as a pyrrole derivative from reaction mixtures containing [α-^{14}C]*glycine.* Unless it is otherwise stated, the reaction mixture, containing the haemolysate or fractionated haemolysate, was incubated with [α-^{14}C]glycine (5 μc, 0·01 M), potassium succinate (0·01 M), the appropriate cofactors, and carrier ALA (100 mg. of the hydrochloride dissolved in 0·25 M-KH_2PO_4–K_2HPO_4 buffer and adjusted to pH 7·4). When systems in which there was no metabolism of ALA were used, the carrier ALA was added at the end of the incubation; with systems which rapidly metabolized ALA, the amount of ALA remaining after incubation was determined by assay with picric acid.

To the reaction mixture after incubation trichloroacetic acid was added to give a final concentration of 5% (w/v), the precipitate of protein was centrifuged off and the supernatant was evaporated to dryness *in vacuo* over NaOH. The residue was extracted once with ether (150 ml.) and the ether extract was discarded. The residue was then dissolved in 0·25 M-Na_2HPO_4 (10 ml.) and the pH was adjusted to 7·0. ALA was then condensed with ethyl acetoacetate to give a pyrrole (Mauzerall & Granick, 1956). Ethyl acetoacetate (1 ml.) was added and the mixture heated in a boiling-water bath for 15 min. The mixture was then cooled and a small amount of precipitate removed by centrifuging. To the supernatant, non-labelled glycine was added and the mixture acidified with conc. HCl to pH 1. The precipitate of 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole which formed was filtered off and washed with a solution of glycine acidified with HCl. It was then dissolved in 2·5 N-NaOH (2 ml.) and the pyrrole again precipitated in the presence of non-labelled glycine by the addition of conc. HCl. The precipitate was filtered off, washed with a small amount of water, dissolved in methanol and transferred to a sublimation flask. After evaporation of the methanol, the pyrrole was sublimed *in vacuo* (water pump) at 160–175° over a period of about 0·5 hr. The sublimate was dissolved in the minimum amount of hot ethyl acetate, the solution was cooled to 0° and light petroleum (b.p. 40–60°) was added. The white crystalline precipitate which formed was filtered off, washed with light petroleum, air-dried and plated for counting.

Estimation of δ-aminolaevulic acid. ALA was estimated colorimetrically by condensation with acetylacetone (Mauzerall & Granick, 1956) or by reaction with picric acid (Shuster, 1956), the sensitivity and specificity of the latter method being increased by the following modifications. The 5% (w/v) trichloroacetic acid filtrate (2·0 ml.) containing up to 0·5 μmole of ALA was mixed with saturated picric acid (0·2 ml.). 2·5N-NaOH (0·5 ml.) was then added and the mixture allowed to stand for 7 min. before the addition of 8N-HCl (1·0 ml.). After 10 min. the excess of picric acid was removed by two extractions with ether (10 ml.) and one extraction with light petroleum (b.p. 40–60°, 10 ml.) and the colour was read in a spectrophotometer (420 mμ) against a reagent blank.

This method allowed ALA to be estimated accurately in the presence of concentrations of α-oxoglutarate up to 0·01 M.

Radioactivity measurements. Counting was done in 1 cm.² disks at infinite thickness with an end-window Geiger counter.

RESULTS

Effect of anaemia on synthesis of haem in vitro

As a system for studying the formation of ALA from glycine and succinate, haemolysates of chicken erythrocytes (Dresel & Falk, 1954) were chosen. The activity of such systems could be greatly increased if the chickens had been previously made anaemic by the injection of phenylhydrazine (Wright & Van Alstyne, 1931; Hammarsten, Thorell, Åqvist, Eliasson & Åkerman, 1953). Haemolysates of these erythrocytes (many of which were immature) synthesized haem from glycine and succinate at a much greater rate than did the haemolysates prepared from normal cells (Table 1).

Fractionation of the haemolysate

In the initial fractionation experiments it was found that on centrifuging the haemolysate (3500 ***g***; 30 min.) the cell solids formed a gelatinous mass which could not readily be resuspended. However, if the haemolysate was homogenized (1–2 min. in a Measuring and Scientific Equipment Ltd. Homogeniser at 0°) before centrifuging, the residue which was then obtained could be resuspended in the supernatant (or in 1·15% KCl), the reconstituted haemolysate having lost only 16% of its activity. In later experiments it was found that by shortening the period of lysis and by keeping the temperature at 0° it was no longer necessary to homogenize the haemolysate before centrifuging. The cell solids obtained in this way could be washed with 1·15% potassium chloride solution with little loss of activity, as measured by incorporation of [α-^{14}C]glycine into haem. When recombined with the original supernatant a 5% drop in activity resulted after two washings. The supernatant alone had only 10% of the activity of the whole haemolysate.

Table 1. *Synthesis of haem in haemolysates from normal and anaemic chickens*

Haemolysates (14 ml.) were incubated in air for 2 hr. at 38° with 12 mM-[α-^{14}C]glycine (2·5 μc), 12 mM-potassium succinate, mM-$MgCl_2$ and 2 mM-$FeCl_3$, final volume of 16 ml. Protoporphyrin methyl ester was prepared from the haemoglobin and its radioactivity determined as described.

Source of haemolysate	Counts of Cu^{2+} protoporphyrin ester/min.
Normal chickens	73
Anaemic chickens	551

However, as a measure of the formation of ALA, a determination of the incorporation of [α-^{14}C]-glycine into haem was unsatisfactory for a number of reasons, and a direct estimate of the amount of ALA synthesized was sought. ALA was rapidly metabolized in the haemolysates and no means of causing its accumulation was known. However, by adding a large excess of carrier ALA before incubation and by re-isolating it at the end of the reaction as 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole (Mauzerall & Granick, 1956) a direct measure of the incorporation of [α-^{14}C]-glycine into ALA could be obtained. Further experiments on the fractionation of the haemolysate showed that the cell solids could be separated into two distinct fractions: an upper, loosely-packed light-coloured fraction (P_1), which could be readily suspended in 1·15% potassium chloride solution; and a lower, tightly-packed dark-coloured fraction (P_2), which was only resuspended with difficulty. These two fractions (P_1 and P_2) were separated and washed, and each was recombined with a portion of the supernatant (S_2). The activity for synthesis of ALA was found to be associated mainly with P_1 (Fig. 1, Table 2). The original supernatant (S_1) was found to have a small amount of activity, but this was lost completely after centrifuging at 105 000 ***g*** for 1 hr.

Further experiments showed that the washed upper layer of particles (P_1) would form ALA from [α-^{14}C]glycine and succinate without the addition of the supernatant, but that the activity was greatly increased in the presence of the whole supernatant (S_2) and to a less extent by a boiled extract or ultrafiltrate of the supernatant (Table 3). This discovery, that the washed particles would synthesize ALA from glycine and succinate, and that the activity could be increased by the addition of heat-stable and diffusible cofactors, was accompanied by the finding that there was no metabolism of ALA by the particles, as shown by the following experiment. When ALA was incubated at 37° with washed particles in the presence of phosphate, there was no disappearance of ALA. Thus a direct

demonstration of a net synthesis of ALA by colorimetric methods was possible. The nature of the heat-labile, non-diffusible material in the supernatant which stimulated the formation of ALA by the particles was not further investigated.

Requirements of particles for the synthesis of δ-aminolaevulic acid

Although the initial experiments on synthesis of ALA by the washed particles were carried out with glycine and succinate as substrates, it was later found that α-oxoglutarate was much more effective than succinate as a precursor of ALA (especially at low concentration), and most of the work reported here was done with α-oxoglutarate as substrate. At a concentration of 24 mM the amount of ALA formed from α-oxoglutarate was twice that formed from succinate, and at a concentration of 1·7 mM six times as much ALA was formed from α-oxoglutarate as from succinate. At low concentrations of α-oxoglutarate, in the presence of non-limiting concentrations of glycine, nearly half of the added α-oxoglutarate was converted into ALA. The optimum concentration of α-oxoglutarate was 1·7 mM, and at high concentrations synthesis of ALA was inhibited (Fig. 2). This inhibition of formation of ALA by excess of substrate was also observed when succinate was used. The washed particles apparently did not contain any endogenous glycine, α-oxoglutarate or succinate, as there was no synthesis of ALA in the presence of cofactors and in the absence of added substrates. There was also an absolute requirement for oxygen, little or no synthesis of ALA occurring under anaerobic conditions with both succinate and α-oxoglutarate (Tables 5 and 6). The conversion of glycine into ALA was less efficient than that of α-oxoglutarate, but there was no inhibition of ALA

Table 2. *Synthesis of δ-aminolaevulic acid in fractionated haemolysates from anaemic chickens*

The fractions were incubated in air for 2 hr. at 38° with 9 mM-[α-^{14}C]glycine (5 μc), 6 mM-potassium succinate, mM-$MgCl_2$ and ALA hydrochloride (100 mg.) in 0·25M-KH_2PO_4–K_2HPO_4 (pH 7·0) (2 ml.). P_1 and P_2 were suspended in 1·15% KCl before addition. The final volume of the reaction mixture was 33 ml. At the end of the reaction the crystalline pyrrole derived from ALA was isolated and its radioactivity determined as described.

Fraction	Counts of 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole/min.
S_1 (20 ml.)	29
S_2 (20 ml.)	0
S_2 (16 ml.) + P_2 (13 ml.)	26
S_2 (16 ml.) + P_1 (13 ml.)	125

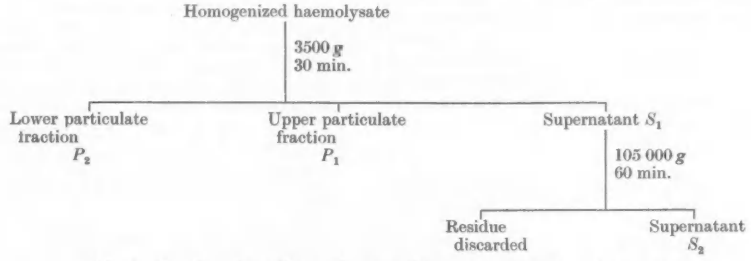

Fig. 1. Fractionation of haemolysates of immature chicken erythrocytes.

Table 3. *Formation of δ-aminolaevulic acid by washed particles (P_1)*

The fractions to be tested were incubated in air at 38° for 2 hr. with 13 mM-[α-^{14}C]glycine (5 μc), 10 mM-potassium succinate, 2 mM-$MgCl_2$, and ALA hydrochloride (100 mg.) in 0·25M-K_2HPO_4–KH_2PO_4 (pH 7·3) (1 ml.). In the first series of experiments 8 ml., and in the second series 10 ml., of washed particles (prepared as described under Methods) were used. The additions made are described in the table. The boiled supernatant was prepared by heating S_2 to 100° for 2 min., centrifuging and taking the supernatant. The ultrafiltrate was prepared by centrifuging S_2 in a cellophan bag supported on a layer of glass beads in a centrifuge tube (3000 *g*; 1 hr.). The crystalline pyrrole derived from ALA was isolated at the end of the reaction and its radioactivity determined.

Expt.	Additions	Series 1 (ml.)	Series 2 (ml.)	Counts of 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole/min. Series 1	Series 2
1	1·15% KCl	10	15	28	38
2	Supernatant (S_2)	10	15	120	126
3	Ultrafiltrate of S_2	10	—	46	—
4	Boiled S_2	—	15	—	52

synthesis by excess of glycine. The optimum concentration of glycine was above 67 mM (Fig. 3) and for most experiments a concentration of 33 mM was used. The effect of concentration of enzyme on formation of ALA is shown in Fig. 4, and the effect of time of incubation in Fig. 5.

The optimum pH for the system with oxoglutarate was 7·2 (Fig. 6). In the presence of borate the optimum pH was similar, but activity was greatly reduced. A similar decrease in activity was also obtained, when phosphate was replaced by tris buffer (Table 4). With succinate as substrate, substitution of phosphate by tris buffer reduced activity by 80–90%, whereas replacement of phosphate by barbitone produced a less severe inhibition. With barbitone the inhibition was almost completely reversed by addition of phosphate, but addition of phosphate to mixtures

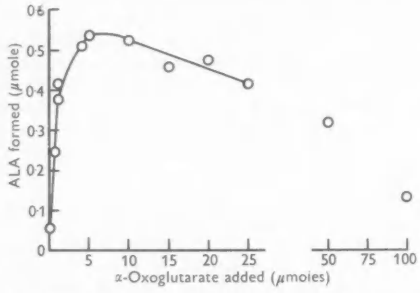

Fig. 2. Effect of concentration of α-oxoglutarate on synthesis of δ-aminolaevulic acid. Particles (1·5 ml.) were incubated in air for 1·5 hr. at 38° with 0·033 M-glycine, 0·0017 M-$MgCl_2$, 0·05 M-K_2HPO_4–KH_2PO_4 (pH 7·4), coenzyme A (0·05 mg.), pyridoxal phosphate (0·05 mg.) and potassium α-oxoglutarate. The volume was made up to 3·0 ml. with 1·5% KCl. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

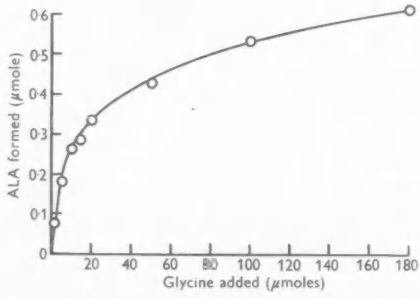

Fig. 3. Effect of concentration of glycine on synthesis of δ-aminolaevulic acid. Particles (1·5 ml.) were incubated in air for 1·5 hr. at 38° with 0·0017 M-potassium α-oxoglutarate, 0·0017 M-$MgCl_2$, 0·05 M-K_2HPO_4–KH_2PO_4 (pH 7·4), coenzyme A (0·05 mg.), pyridoxal phosphate (0·05 mg.) and glycine. The volume was made up to 3·0 ml. with 1·15% KCl. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

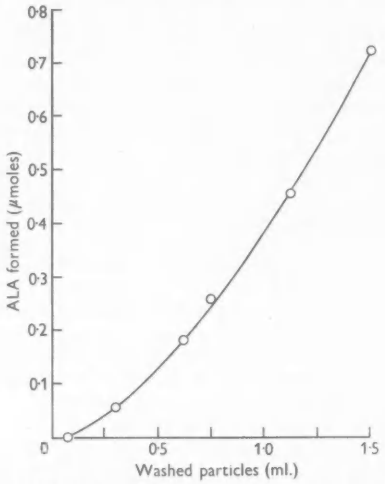

Fig. 4. Effect of concentration of enzyme on formation of δ-aminolaevulic acid. Particles were incubated in air for 1·5 hr. at 38° with 0·0017 M-potassium α-oxoglutarate, 0·033 M-glycine, 0·0017 M-$MgCl_2$, 0·05 M-KH_2PO_4–K_2HPO_4 (pH 7·4), coenzyme A (0·05 mg.) and pyridoxal phosphate (0·05 mg.). The volume was made up to 3·0 ml. with 1·15% KCl. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

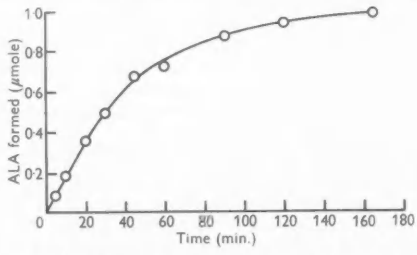

Fig. 5. Effect of time of incubation on formation of δ-aminolaevulic acid. Particles (1·44 ml.) were incubated in air at 38° with 0·0017 M-potassium α-oxoglutarate, 0·033 M-glycine, 0·0017 M-$MgCl_2$, 0·05 M-KH_2PO_4–K_2HPO_4 (pH 7·4), coenzyme A (0·05 mg.) and pyridoxal phosphate (0·05 mg.). The volume was made up to 3·0 ml. with 1·15% KCl. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

containing tris buffer resulted in only 50% reactivation (Table 4).

The effects of various cofactors on the formation of ALA from glycine and oxoglutarate or succinate by the particles are shown in Tables 5 and 6. Duplicates for experiments carried out with the same enzyme preparations agreed within ±4% but different preparations did not behave in an identical manner with regard to activation by pyridoxal phosphate, coenzyme and $MgCl_2$. The omission of coenzyme A, pyridoxal phosphate or magnesium chloride caused a decrease in synthesis of ALA by the particles. The effects of other substances are shown in Table 7. Ethylenediaminetetra-acetic acid (EDTA) in the absence of magnesium also caused a considerable activation. Cyanide and the sulphydryl reagents, *p*-chloromercuribenzoate and iodoacetamide were very strongly inhibitory, but 2:4-dinitrophenol and sodium azide in concentrations which are stated to uncouple oxidative phosphorylation completely (Hunter, 1955) were virtually without effect. Glutathione, in both the presence and the absence of coenzyme A, was slightly inhibitory and nicotinamide and sodium fluoride were without significant effect.

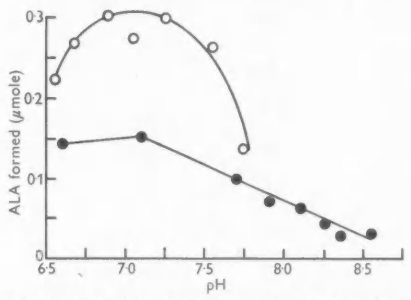

Fig. 6. Effect of pH on formation of δ-aminolaevulic acid. Particles (1·0 ml.) were incubated in air at 38° for 1·5 hr. with 0·017 M-potassium α-oxoglutarate, 0·033 M-glycine, 0·0017 M-$MgCl_2$, coenzyme A (0·05 mg.), pyridoxal phosphate (0·05 mg.) and 0·05 M-KH_2PO_4–K_2HPO_4 (○) or a mixture of 0·05 M-boric acid–potassium borate and 0·01 M-KH_2PO_4–K_2HPO_4 (●). The volume was made up to 3 ml. with 1·15% KCl. The pH of the reaction mixtures were checked with the glass electrode before incubation. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

The effects of other substances on the formation of ALA from glycine and succinate or α-oxoglutarate was also studied. Under aerobic conditions and in the presence of phosphate the addition of DPN, TPN, glucose, glucose 6-phosphate, fructose 1:6-diphosphate, D-3-phosphoglycerate and ATP produced very slight and variable results. In the absence of oxygen DPN had a slight, but definite, activating effect.

Effect of penicillamine

Because of the finding of du Vigneaud, Kuchinskas & Horvath (1957) that L-penicillamine inhibited rat-liver alanine–glutamic and aspartic–glutamic transaminase activities *in vitro* and that this inhibition could be reversed by the addition of pyridoxal phosphate, the effect of L-penicillamine and D-penicillamine on the synthesis of ALA by the washed particles was investigated. The results

Table 4. *Effects of various buffers on synthesis of δ-aminolaevulic acid from glycine and succinate or α-oxoglutarate by washed particles*

The system contained suspension of particles (1 ml.), 16 mM-potassium succinate or 16 mM-potassium α-oxoglutarate, 40 mM-glycine, 2 mM-$MgCl_2$, coenzyme A (0·05 mg.) and pyridoxal phosphate (0·1 mg.). The buffers used were: (*a*) 0·05 M-KH_2PO_4–K_2HPO_4, pH 7·4; (*b*) sodium barbitone–HCl, pH 7·4; (*c*) 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl, pH 7·6. The incubations were carried out at 38° for 1 hr. in air.

Expt. no.	Substrate	Buffer	Activity (μmole of ALA/ml. of particles/hr.)
1	Succinate	0·05 M-Phosphate	0·077
	Succinate	0·05 M-Tris	0·010
2	Succinate	0·05 M-Phosphate	0·12
	Succinate	0·05 M-Barbitone	0·07
3	Succinate	0·05 M-Phosphate	0·14
	Succinate	0·05 M-Barbitone	0·04
	Succinate	0·05 M-Barbitone + 0·05 M-phosphate	0·12
4	Succinate	0·05 M-Phosphate	0·11
	Succinate	0·05 M-Tris	0·013
	Succinate	0·05 M-Tris + 0·05 M-phosphate	0·063
5	Succinate	0·05 M-Phosphate	0·091
	Succinate	0·05 M-Tris	0·021
	α-Oxoglutarate	0·05 M-Phosphate	0·174
	α-Oxoglutarate	0·05 M-Tris	0·071

Table 5. *Cofactor requirements for synthesis of δ-aminolaevulic acid from glycine and α-oxoglutarate by washed particles*

Control tubes contained particles, 17 mM-potassium α-oxoglutarate, 33 mM-glycine, 17 mM-$MgCl_2$, 50 mM-KH_2PO_4–K_2HPO_4 (pH 7·4), coenzyme A (0·05 mg.) and pyridoxal phosphate (0·05 mg.). The volume was made up to 3·0 ml. with 1·15% KCl. The tubes were incubated in air at 38° for 1·5 hr. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid. Substances omitted from the control are shown below.

Omissions	Activity (μmole of ALA/ml. of packed particles/1·5 hr.) Test system	Control system	Test system as percentage of control (averaged where applicable)
Pyridoxal phosphate	0·300	0·359	83
Pyridoxal phosphate and coenzyme A	0·211	0·359	59
Coenzyme A	0·303	0·359	84
	0·474	0·520	91 } 85
	0·647	0·799	81
$MgCl_2$	0·435	0·570	76
	0·522	0·799	65 } 71
Air	0·014	0·432	3
	0·057	0·799	7 } 5

Table 6. *Cofactor requirements for synthesis of δ-aminolaevulic acid from glycine and succinate by washed particles*

The complete system contained suspension of particles (1 ml.), 16 mM-potassium succinate, 40 mM-glycine, 2 mM-$MgCl_2$, 50 mM-KH_2PO_4–K_2HPO_4 (pH 7·4), coenzyme A (0·05 mg.) and pyridoxal phosphate (0·1 mg.). The volume was made up to 2·5 ml. with 1·15% KCl. The tubes were incubated at 38° for 1 hr. Omissions are indicated below. The reaction was stopped by adding 20% trichloroacetic (1 ml.) and ALA estimated by the picric acid method.

Omissions	Activity (μmole of ALA/ml. of particles/hr.) Test system	Control system	Test system as percentage of control
Pyridoxal phosphate	0·065	0·080	81
Coenzyme A	0·052	0·080	65
Coenzyme A	0·11	0·16	69
$MgCl_2$	0·026	0·080	33
$MgCl_2$	0·10	0·16	63
Succinate	0·000	0·080	0
Succinate	0·03	0·16	17
Air	0·015	0·14	11

(Table 8) showed that L-penicillamine caused a marked decrease in the amount of ALA formed from glycine and α-oxoglutarate, whereas D-penicillamine had only a slight effect. du Vigneaud *et al.* (1957) had also observed that D-penicillamine had only slight activity as inhibitor on transaminases compared with the L-isomeride. The addition of increasing amounts of pyridoxal phosphate to the reaction mixture containing L-penicillamine caused a slight reversal of this inhibition. The reversing effect of pyridoxal phosphate is slight and is smaller than that found by du Vigneaud *et al.* (1957) for transaminase.

Effect of S-succinyl-coenzyme A

Succinyl-CoA was prepared from 5 mg. of coenzyme A and 0·43 mg. of succinic anhydride according to Simon & Shemin (1953). A solution containing nominally 1·5 μmoles/ml. was used; on incubation at 0° for 30 min. of 0·5 ml. of this solution with 0·1 ml. of neutralized 2·4 M-hydroxylamine hydrochloride a strongly positive hydroxamic acid reaction with ferric chloride was obtained. A control sample containing succinic anhydride, but no CoA, gave a negative hydroxamic acid reaction under these conditions. Incubation of a suspension of particles (1 ml.) with succinate, glycine, magnesium chloride, pyridoxal phosphate and coenzyme A under standard conditions (Table 6) gave an ALA synthesis of 1·8 μmoles/ml.; addition of succinyl-CoA solution (0·5 ml.) for this system resulted in a slight reduction in synthesis of ALA (0·165 μmole/ml.). When succinate and coenzyme A were replaced by succinyl-CoA there was no significant amount of ALA synthesis. In another experiment there was slight synthesis of ALA under such conditions.

Decarboxylation of glycine by the washed particles

In order to investigate the association of the decarboxylation of glycine with its condensation with the active succinyl derivative, the liberation of radioactive carbon dioxide from [*carboxy*-^{14}C]-glycine by the particles was examined in the presence and absence of α-oxoglutarate.

Table 7. *Effects of various substances on the synthesis of δ-aminolaevulic acid from glycine and α-oxoglutarate by washed particles*

Control tubes contained particles, 0·0017 M-potassium α-oxoglutarate, 0·033 M-glycine, 0·0017 M-$MgCl_2$, 0·05 M-KH_2PO_4–K_2HPO_4 (pH 7·4), coenzyme A (0·05 mg.) and pyridoxal phosphate (0·05 mg.). The volume was made up to 3·0 ml. with 1·15% KCl. The tubes were incubated in air at 38° for 1·5 hr. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid. Substances added to the control are shown below.

Additions	Activity (μmole of ALA/ml. of packed particles/1·5 hr.) Test system	Control system	Test system as percentage of control
KCN (0·5 mM)	0·000	0·520	0
KCN (0·1 mM)	0·122		23
KCN (0·01 mM)	0·467		90
2:4-Dinitrophenol (0·33 mM)	0·446		86
2:4-Dinitrophenol (0·66 mM)	0·419		81
2:4-Dinitrophenol (mM)	0·426		82
Sodium azide (2 mM)	0·467		90
Sodium azide (4 mM)	0·412		79
Sodium azide (6 mM)	0·412		79
$CaCl_2$ (0·2 mM)	0·419		81
Glutathione (10 mM)	0·495		95
Sodium fluoride (10 mM)	0·537		103
Sodium fluoride (mM)	0·579		111
EDTA (mM; $MgCl_2$ omitted)	0·826	0·522*	158
EDTA (0·25 mM; $MgCl_2$ omitted)	0·840		161
p-Chloromercuribenzoate (mM; CoA omitted)	0·016	0·647†	2
p-Chloromercuribenzoate (0·25 mM; CoA omitted)	0·064		10
Iodoacetamide (2 mM; CoA omitted)	0·164		25
Iodoacetamide (0·5 mM; CoA omitted)	0·340		53
Nicotinamide (10 mM)	0·624	0·605	103
None	—	0·392‡	—

* $MgCl_2$ omitted from control system.
† Coenzyme A omitted from control system.
‡ ALA estimated by condensation with acetylacetone (pyridoxal phosphate omitted in this experiment).

Table 8. *Effect of* L-*penicillamine and* D-*penicillamine on the formation of δ-aminolaevulic acid*

Each tube contained particles, 0·0017 M-potassium α-oxoglutarate, 0·033 M-glycine, 0·0017 M-$MgCl_2$, 0·05 M-KH_2PO_4–K_2HPO_4 (pH 7·4) and coenzyme A (0·05 mg.), and the additions made are shown below. The volume was made up to 3·0 ml. with 1·15% KCl. The tubes were incubated at 38° for 1·5 hr. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

Expt. no.	Additions	μmole of ALA/ml. of packed particles/1·5 hr.	Inhibition (%)
1	None	0·389	—
	L-Penicillamine (2·67 mM)	0·071	82
	L-Penicillamine (2·67 mM) + pyridoxal phosphate (0·134 mM)	0·081	79
	L-Penicillamine (2·67 mM) + pyridoxal phosphate (0·268 mM)	0·094	76
	D-Penicillamine (2·67 mM)	0·333	15
2	None	0·513	—
	L-Penicillamine (2·25 mM)	0·086	83
	L-Penicillamine (2·25 mM) + pyridoxal phosphate (0·95 mM)	0·145	72

Table 9. *Decarboxylation of* [carboxy-^{14}C]*glycine by particles in the presence and absence of α-oxoglutarate*

Flasks contained washed particles (15 ml.), [*carboxy*-^{14}C]glycine (5 μc, 38·5 mg.), coenzyme A (0·5 mg.), pyridoxal phosphate (0·5 mg.), 0·002 M-$MgCl_2$, 0·05 M-KH_2PO_4–K_2HPO_4 (pH 7·4), and $NaHCO_3$ (5 mg.). The volume was made up to 30 ml. with 1·15% KCl. The additions made are shown below.

A slow stream of CO_2-free air was passed through the reaction mixture during incubation for 1·5 hr. at 38°, and the CO_2 evolved in the reaction was collected in two 25 ml. portions of CO_2-free N-NaOH.

At the end of the incubation $NaHCO_3$ (10 mg.) in water (1 ml). was injected into the reaction flask through the rubber tubing, followed by 3 N-HCl (6 ml.). Air was passed through the system for a further 10 min., and then 5 M-NH_4Cl (6 ml.) was injected into each of the sodium hydroxide vessels. The contents of these two vessels were then mixed and an excess of $BaCl_2$ was added. The $BaCO_3$ precipitate was filtered off, washed with water and acetone and dried, and its radioactivity determined. At the end of the experiment carrier ALA (100 mg.) was added to flask 2 and re-isolated by reaction with acetoacetate and its radioactivity determined as described under Methods.

The amount of ALA synthesized by the particles under these conditions was estimated in a parallel small-scale experiment by assay with picric acid.

Additions	μmole of ALA formed	Counts of barium carbonate/min.	Counts of 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole/min.
Flask 1 None	0·13	257	—
Flask 2 1·7 mM-potassium α-oxoglutarate	7·89	2227	15

The washed particles were incubated with [*carboxy*-^{14}C]glycine and the carbon dioxide evolved was collected as barium carbonate and its radioactivity determined. The ALA formed was also measured. The results showed that in the presence of α-oxoglutarate there was nine times as much labelling in the carbon dioxide as when α-oxoglutarate was not added (Table 9).

Synthesis of δ-aminolaevulic acid by particles obtained from non-anaemic chickens

Particles obtained from lysates of erythrocytes from normal chickens were tested for their capacity to form ALA from glycine and α-oxoglutarate or succinate by using the same methods as those employed when the cells from phenylhydrazine-treated birds were used. With glycine and α-oxoglutarate as substrates, 0·060 μmole of ALA/ml. of particles/1·5 hr. was formed, as compared with a value of 0·547 μmole of ALA/ml. of particles/1·5 hr. obtained with particles from phenylhydrazine-treated birds (average from six experiments). With glycine and succinate as substrates, 0·003 μmole of ALA/ml. of particles/hr. was formed with non-anaemic birds, as compared with a value of 0·120 μmole of ALA/ml. of particles/hr. obtained with birds which had been treated with phenylhydrazine (average of nine preparations).

DISCUSSION

The experiments reported in the present paper show that particles obtained from the lysates of erythrocytes of chickens, which had been made anaemic, can synthesize ALA from glycine and succinate or α-oxoglutarate. The identification of the product of the enzymic reaction as ALA rests on the following observations. The material gives with picric acid a colour having the same spectral characteristics and under the same conditions as does ALA; the colour, like that produced with authentic ALA, is partially extracted into ethyl acetate. The substance produced by incubation also gives, after condensation with acetylacetone or ethyl acetoacetate, the pyrrole reaction with Ehrlich's reagent. Observations that are reported in Part 2 (Gibson, Laver & Neuberger, 1958*b*) show quantitative agreement for the amount of ALA produced in enzymic experiments, if measured by the modified method of Shuster (1956) or by the pyrrole method of Mauzerall & Granick (1956). Moreover, when the system was incubated with [α-^{14}C]glycine and non-radioactive succinate and a large amount of non-radioactive ALA was then added, a crystalline pyrrolecarboxylic acid was obtained, after condensation with ethyl acetoacetate, which was radioactive (Table 3). In another experiment, not reported in detail, the incubation was done with non-radioactive glycine and radioactive succinate; carrier ALA was again added and the ALA was isolated after chromatography on paper with butanol–acetic acid as solvent. The aminolaevulic acid hydrochloride was recrystallized several times to constant radioactivity and repeatedly extracted with ether to remove possible traces of succinic acid. On oxidation with periodate (Shemin *et al.* 1955) radioactive succinic acid was produced. Evidence showing that the formation of the substance behaving as ALA is associated with the decarboxylation of glycine was also obtained (Table 9). These observations taken together provide convincing evidence that ALA is indeed formed. But these experiments do not indicate whether ALA is the initial product of the condensation or whether it arises by enzymic or spontaneous decarboxylation (W. G. Laver, A. Neuberger & J. J. Scott, unpublished work) of α-amino-β-oxoadipate.

The fraction which was found to be active in the synthesis of ALA contains nuclei which can be seen under the microscope, and probably also cell debris arising from the cell membrane. On differential centrifuging of the supernatant fluid no evidence could be obtained that the erythrocytes contain mitochondria. The active particulate fraction is rich in deoxyribonucleic acid but also contains some ribonucleic acid. No conclusion can at present be drawn as to whether the enzymic activity is associated with nuclei, the cell membrane or some other cell constituents, such as mitochondria, which for unknown reasons may be obtained in the 'nuclear' fraction. Rubinstein & Denstedt (1953, 1954) found that in lysates of erythrocytes from non-anaemic chickens the succinic dehydrogenase, succinic oxidase and cytochrome oxidase activities are associated with nuclei. These authors also comment on the apparent absence of mitochondria in these cells.

The particles obtained by lysis of the red cells can synthesize ALA, provided that glycine, succinate or α-oxoglutarate and oxygen are supplied. There is no absolute requirement for phosphate, but the amount of ALA produced is markedly or even greatly decreased if phosphate is replaced by tris, barbitone or borate buffer (Table 4, Fig. 6). Addition of magnesium chloride increased the formation of ALA from oxoglutarate (Table 5) by about 50% and to an even greater extent from succinate (Table 6). The addition of pyridoxal phosphate and of coenzyme A, both separately and together, activated the formation of ALA. The effects were not very large, the activation varying between 15 and 50%; but they were consistently found in a large number of experiments. The belief that these two substances are cofactors for the enzymic reactions studied in the particles is based mainly on circumstantial evidence. The marked inhibition by L-penicillamine observed here is similar to that observed by du Vigneaud *et al.* (1957) with a liver transaminase which is known to be an enzyme containing pyridoxal phosphate as prosthetic group. When the particles are broken up by freeze-drying or homogenization, succinyl-CoA, but not α-oxoglutarate or succinate, can be used as substrate for the formation of ALA (Gibson, Laver & Neuberger, 1958*a*, *b*). It seems reasonable to assume that succinyl-CoA is also the active intermediate in intact particles. With whole particles, however, succinyl-CoA was not active. This must be ascribed to the existence of permeability barriers or to the action of a deacylase.

The requirement of pyridoxal phosphate for haem synthesis and the effect of pantothenic acid deficiency have been investigated by Schulman & Richert (1957). These authors obtained evidence that pyridoxal phosphate is concerned in the formation of ALA and they also suggest that the effect of pantothenic acid is explicable in terms of a requirement for coenzyme A. Similar results have been reported on cofactor requirements for porphyrin synthesis in *Tetrahymena vorax* by Lascelles (1957). In this organism synthesis of porphyrin from glycine is greatly reduced by deficiency of pyridoxal or pantothenate, whereas synthesis from ALA is not affected. The inhibitory effect of *p*-chloromercuribenzoate and iodoacetamide is also readily explained, if CoA is a necessary cofactor in the condensation. The action of the other inhibitors and activators and the detailed mechanism of the reaction are discussed in Part 2.

SUMMARY

1. Particles have been obtained, from lysates of erythrocytes of anaemic chickens, which catalyse a net synthesis of δ-aminolaevulic acid from glycine and succinate or α-oxoglutarate. δ-Aminolaevulic acid was not further metabolized by these particles. Preparations from non-anaemic chickens had little or no activity.

2. Synthesis of δ-aminolaevulic acid required the addition of glycine, succinate or α-oxoglutarate and the presence of oxygen, and was increased by the addition of pyridoxal phosphate, coenzyme A, phosphate and magnesium chloride. Inhibition of synthesis of δ-aminolaevulic acid was produced by addition of cyanide, iodoacetamide and *p*-chloromercuribenzoate. Ethylenediaminetetra-acetic acid showed a marked stimulating action. A number of other substances such as sodium azide, 2:4-dinitrophenol and sodium fluoride had no significant effect.

3. The pH optimum was found to be near 7·2, when phosphate buffer was used. Replacement of phosphate by borate, barbitone or 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer reduced the amount of δ-aminolaevulic acid formed.

4. L-Penicillamine, but not D-penicillamine, was strongly inhibitory. It is suggested that this inhibition is caused by interaction with pyridoxal phosphate.

5. No δ-aminolaevulic acid was formed when succinate or α-oxoglutarate was replaced by synthetic succinyl-coenzyme A. It is suggested that this is the result of permeability barriers.

6. The decarboxylation of glycine by the particles was found to be greatly increased by addition of α-oxoglutarate.

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The Association of Adrenaline and Noradrenaline with Blood Platelets

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It is becoming apparent that blood platelets, in addition to their classical role in blood coagulation, have other important functions. Among these their function as carriers of pharmacologically active amines is of particular interest. Weil-Malherbe & Bone (1954*a*) have briefly reported that platelets contain adrenaline and noradrenaline, and more extensive data will now be presented. Experiments on the nature of this association will also be described.

The results were obtained with a fluorimetric method which, though not strictly specific for adrenaline and noradrenaline, is believed to be specific for the catechol grouping. Of other biologically occurring catechol compounds those possessing a carboxylic or ketonic group in the side chain give rise to much smaller fluorescence intensities than adrenaline and noradrenaline and would interfere only if they were present in great excess. Experiments with ion-exchange resins, paper chromatography and paper electrophoresis have shown that interfering substances do not significantly contribute to the fluorescence obtained when the method is applied to plasma (Weil-Malherbe & Bone, 1957*b*). Although we have so far been unable to demonstrate the presence of adrenaline by bioassay in plasma of resting human subjects, Born & Hornykiewicz (1957) were successful in demonstrating the presence of adrenaline, though not noradrenaline, by bioassay in pig platelets.

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METHODS

Collection of blood

When human blood was used, the donors were hospital patients suffering from mental disorders. Blood was collected from an antecubital vein with a transfusion needle (16 S.W.G.) mounted on a short length of polyvinyl chloride tubing and was run into a measuring cylinder containing anticoagulant solution. Unless stated otherwise, a solution containing 1% (w/v) of neutralized sodium ethylenediaminetetra-acetate (EDTA) and 2% (w/v) of $Na_2S_2O_3,5H_2O$ (Renton & Weil-Malherbe, 1956) was used in the proportion of 1 vol to 3 vol. of blood. This anticoagulant is referred to below as 'EDTA–thio solution'. Solid heparin, containing 107 units/mg. (Evans Medical Supplies Ltd.), was dissolved in a minimum volume of 0·9% NaCl and

used in the proportion of 200 units/10 ml. of blood. The composition of other anticoagulants is indicated in the tables.

Preparation of platelets

Blood was first centrifuged at about 600 *g* for 20 min. at room temperature; the supernatant ('platelet-rich plasma') was centrifuged a second time in the angle head, no. 6886, of the MSE refrigerator centrifuge (Measuring and Scientific Equipment Ltd., London) at 4000 rev./min. (about 2500 *g*) for 20 min. at 10°. The plasma ('platelet-poor') was thoroughly drained off and the deposit of platelets redistributed in a mixture of 3 vol. of 0·9% NaCl and 1 vol. of EDTA–thio, equal in volume to the original volume of plasma. The platelets were washed once or twice by centrifuging and suspension in the same solution; washing was sometimes omitted, especially in many of the estimations included in Table 2.

Estimation of adrenaline and noradrenaline

The platelet suspension, prepared from 15–20 ml. of blood, was adjusted to pH 8·5 with 0·5N-Na_2CO_3 solution and passed, under pressure (40 mm. Hg), through a column of 0·7 g. of alumina (5 mm. × 45 mm.) previously washed with 5 ml. of 0·2M-sodium acetate. Details of the technique have been described by Weil-Malherbe & Bone (1952). Filtration usually proceeded smoothly and the platelet suspension emerged apparently unchanged, macro- and micro-scopically, from the column, although comparative platelet numbers were not determined. Plasma, whether platelet-rich or platelet-poor, was treated as described by Weil-Malherbe & Bone (1952), i.e. it was diluted with an equal volume of sodium acetate buffer before filtration through alumina. The adsorbed catecholamines were eluted from the column and estimated by the differential fluorimetric method (Weil-Malherbe & Bone, 1952, 1953).

Concentrations of catecholamines in platelet-poor plasma and in platelets were calculated relative to the volume of platelet-rich plasma from which these fractions were prepared.

Platelet counts

For counts on whole blood the method of Brecher & Cronkite (1950) was used. Counts on platelet-rich plasma were carried out after 100-fold dilution with the following solution: 1 g. of EDTA (disodium salt), 0·7 g. of NaCl, 0·1 g. of Brilliant Cresyl Blue, 3 ml. of N-NaOH, in 100 ml. Platelets in 1 mm.² (400 small squares) of the improved Neubauer chamber were counted in each determination.

RESULTS

Distribution of adrenaline and noradrenaline between plasma and platelets

When 'platelet-rich' plasma was centrifuged, analysis showed the presence of catecholamines both in the 'platelet-poor' supernatant and in the deposit of platelets. As shown in Table 1, the sums of the catecholamine concentrations in the two fractions account, within the limits of error, for the concentrations in the unfractionated sample.

The data shown in Table 2 represent two series of estimations. The first of these was carried out between June 1954 and July 1955, the second between November 1956 and May 1957; in the interval, modifications of the fluorimeter were introduced (cf. Weil-Malherbe & Bone, 1957*a*), which may be responsible for the fact that the absolute concentrations were lower by about one-third in the second than in the first series, the differences being significant. Although there was a considerable scatter in individual samples, adrenaline and noradrenaline were both about evenly distributed between plasma and platelets in both series of estimations on the average. The distribution of adrenaline between plasma and platelets correlated with that of noradrenaline; the

Table 1. *Distribution of catecholamines in human plasma and platelets*

Details are given under Methods. The values represent means ± S.E.M. of six different samples.

	Adrenaline	Noradrenaline
	(μg./l. of plasma)	
(*a*) Plasma, platelet-rich	2·27 ± 0·27	8·39 ± 1·58
(*b*) Plasma, platelet-poor	0·88 ± 0·16	3·70 ± 0·23
(*c*) Platelets	1·18 ± 0·17	3·71 ± 0·33
Sum (*b* + *c*)	2·06 ± 0·31	7·41 ± 0·43

Table 2. *Concentrations of adrenaline and noradrenaline in plasma and platelets*

Values are means ± S.E.M. (with range). For details see text.

Series	No. of observations		Plasma (μg./l.)	Platelets (μg./l. of platelet-rich plasma)	Percentage in platelets of total adrenaline or noradrenaline
I	85	Adrenaline	1·11 ± 0·056 (0·42–2·80)	1·18 ± 0·047 (0·27–2·73)	51·95 ± 1·06 (21·6–70·3)
		Noradrenaline	3·76 ± 0·115 (1·74–6·36)	4·28 ± 0·158 (1·63–8·25)	52·66 ± 0·76 (35·1–70·8)
II	31	Adrenaline	0·74 ± 0·068 (0·39–2·41)	0·82 ± 0·058 (0·30–1·59)	52·6 ± 1·96 (28·5–73·6)
		Noradrenaline	2·60 ± 0·261 (1·17–9·40)	2·72 ± 0·236 (1·20–5·92)	50·9 ± 2·17 (24·2–72·6)

coefficient of correlation, calculated on the combined total of 116 samples, was 0·602.

Platelet counts on platelet-rich plasma were available for many of the samples in the first series and all those in the second series of thirty-one estimations. To facilitate comparison with the results of other authors who determined the concentrations of histamine (Graham, Lowry, Wheelwright, Lenz & Parish, 1955; Humphrey & Jaques, 1954) and serotonin (Humphrey & Jaques, 1954; Humphrey & Toh, 1954; Zucker & Borrelli, 1955, 1956) concentrations were expressed in μmg./10^9 platelets. The mean contents of adrenaline and noradrenaline/10^9 platelets ($\pm$S.E.M.) were $1{\cdot}92 \pm 0{\cdot}13$ μmg. (range 1·07–3·82 μmg.) and $6{\cdot}01 \pm 0{\cdot}48$ μmg. (range 2·81–14·6 μmg.) respectively. The concentration of catecholamines in platelets is thus about one-hundredth of that of serotonin (Zucker & Borrelli, 1955). From data collected by Maupin (1954) it appears that, for a count of 500 000/μl. of plasma, the platelet volume accounts for 0·85% of the volume of plasma. Assuming an equal distribution of catecholamines between platelets and plasma and allowing for dry matter [12·5 and 7% (w/w) respectively] it may be calculated that the concentration of catecholamines in platelets is about 125 times higher than in plasma.

Effects of collection procedures

The vulnerability of platelets is well known; contact with glass seems to be particularly injurious to them. It is therefore common practice to use silicone-coated glassware in experiments with platelets. However, as shown in Table 3, the use of untreated glassware did not appreciably affect the distribution of catecholamines between plasma and platelets. To exaggerate the effect of glass surfaces a pinch of powdered glass was thoroughly mixed with blood before centrifuging in one experiment, but not even this procedure led to a marked release of platelet-bound catecholamines. In most of the later experiments therefore untreated glassware was used.

The choice of anticoagulant solutions did not significantly alter the catecholamine content of plasma, but it had a marked effect on that of the platelet fraction (Table 4). The data for the platelet counts given in Table 4 show that this difference was due to the effect of the anticoagulant on the number of platelets in plasma. The amount of catecholamines per unit number of platelets was hardly affected by a change of anticoagulant.

Platelet yields were highest with EDTA as anticoagulant, whereas they were low with oxalate or fluoride, clumping being observed microscopic-

Table 3. *Effect of glass on distribution of catecholamines between plasma and platelets*

In Expt. 1 about 0·5 g. of powdered glass was added to blood collected in uncoated glassware and the mixture was gently inverted several times before being centrifuged. Standard anticoagulant.

		Plasma		Platelets	
		Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
Expt. no.	Treatment of glassware	(μl./l. of plasma)			
1	Uncoated	0·58	2·94	0·62	3·70
	Silicone-coated	0·43	2·78	0·81	3·81
2	Uncoated	0·82	4·80	1·07	6·90
	Silicone-coated	0·91	4·10	1·44	7·00

Table 4. *Effect of different anticoagulants on platelet count and concentration of catecholamines*

P represents vol. of anticoagulant solution mixed with 10 vol. of blood. A = adrenaline, N = noradrenaline.

				Plasma		Platelets			
				A	N	A	N	A	N
Expt. no.	Anticoagulant	*P*	No. of platelets/μl. of platelet-rich plasma	(μg./l. of plasma)				(μmg./10^9 platelets)	
1	EDTA–thio	3·3	503 000	0·63	1·51	0·82	1·65	1·63	3·28
	Heparin	—	265 000	0·66	1·20	0·59	0·90	2·22	3·40
2	Sodium EDTA, 2·5% (w/v)	2·0	614 000	0·63	1·90	0·79	2·37	1·29	3·85
	Potassium oxalate, 1·8% (w/v)	1·5	355 000	1·06	2·70	0·42	1·43	1·18	4·03
3	Sodium EDTA, 2·5% (w/v)	1·75	540 000	0·56	1·56	0·67	2·11	1·24	3·92
	Sodium citrate, 3·8% (w/v)	1·75	420 000	0·45	1·56	0·45	1·50	1·07	3·58
	Sodium oxalate, 1·3% (w/v)	1·75	280 000	0·67	1·50	0·33	1·11	1·18	3·96

Table 5. *Retention of catecholamines by platelets during blood clotting*

Anticoagulant was added to a portion of a blood sample and the other portion was allowed to clot. Catecholamines were then determined in the serum and in the plasma after removal of platelets by centrifuging.

Expt. no.	Anticoagulant	Fraction	Adrenaline (μg./l.)	Noradrenaline (μg./l.)
1	Heparin	Plasma	0·50	4·08
	None	Serum	0·45	3·52
2	EDTA–thio	Plasma	0·80	3·32
	None	Serum	0·73	3·50
3	EDTA–thio	Plasma	0·45	2·20
	None	Serum	0·36	1·60
	EDTA–thio	Serum*	0·51	2·28

* Clotting was induced by addition of 50 units of thrombin to 40 ml. of platelet-rich plasma.

Table 6. *Release of adrenaline (A) and noradrenaline (N) from platelets by lytic procedures*

Platelet samples used in any one experiment were prepared from the same sample of platelet-rich plasma; each platelet sample corresponded to 20 ml. of blood.

Expt. no.		μg./l. of plasma A	μg./l. of plasma N	% of content of untreated platelets A	% of content of untreated platelets N
1 (means of three experiments)	Platelets, untreated	0·84	3·77	—	—
	After freezing:				
	Insol. residue	0·37	1·40	44·0	37·2
	Supernatant	0·40	1·62	47·6	43·0
2	Platelets, untreated	0·67	2·60	—	—
	After suspension in 0·2% cetyltrimethylammonium bromide:				
	Supernatant	0·25	1·03	37·3	39·6
3	Platelets, untreated	1·07	6·90	—	—
	After extraction with butanol:				
	In extract	0·93	7·10	87	103

ally. Heparin and citrate gave intermediate yields. Silicone-coated glassware was used in these experiments, but in spite of this clumps of platelets were found to adhere to the walls of centrifuge tubes when oxalate or fluoride was used. When heparinized blood was cooled in ice clumping was very marked and, on centrifuging, a practically platelet-free plasma containing less than 1% of the original number of platelets was obtained. A similar observation relating to the effect of oxalate and citrate on platelet counts in plasma has been reported by Barkhan (1957).

Effect of clotting

In marked contrast with serotonin, which is partly released from platelets during the clotting process (Zucker & Borrelli, 1955; Hardisty & Stacey, 1955), catecholamines are not liberated, as shown by the fact that their concentration is the same in centrifuged plasma as in serum, whether formed by spontaneous or by thrombin-induced coagulation (Table 5).

Extraction of platelet-bound catecholamines

As described under Methods the catecholamine content of the platelet suspension was estimated by passing it through a column of alumina at pH 8·5. Catecholamines are quantitatively extracted by this procedure. This was shown in two ways: (1) after the platelet suspension had been passed a second time through a column of alumina, no further fluorescence-producing material could be eluted from the column; (2) when the platelet suspension, after filtration through the column, was centrifuged and the precipitate extracted with acid methanol (Montagu, 1956) no catecholamines were found.

The question arises whether adrenaline and noradrenaline are held within the platelets by a membrane-like structure or whether they are combined with insoluble constituents. If they are held by a membrane, lytic procedures would release them. Saline suspensions of platelets were therefore frozen at $-40°$ for 20 min. and thawed at 37°.

After centrifuging, the residue and supernatant were analysed separately. The initial catecholamine content of the platelets was determined on an untreated portion of the suspension. In another experiment cetyltrimethylammonium bromide, a surface-active agent, was added to a platelet suspension to give a final concentration of 0·2%. The mixture was left at room temperature for 20 min. and centrifuged. Only the supernatant was analysed, since the residue could not be redispersed sufficiently to be passed through an alumina column. Finally a platelet suspension was extracted three times with 2 vol. of butanol and the extract was analysed as described by Weil-Malherbe & Bone (1954*b*).

The results are shown in Table 6. Less than half of the catecholamines in platelets are released by lysis, whether brought about by freezing and thawing or by surface activity. Extraction with butanol, on the other hand, was practically quantitative.

Stability of platelet-bound catecholamines in vitro

When platelet-rich plasma was incubated at 37° for 2 hr. in CO_2+O_2 (5:95, v/v), a considerable disappearance of catecholamines was often noticed. The loss was always larger in platelets than in plasma and greatly depended on the anticoagulant used. The different effects of anticoagulants are shown in Table 7; platelet-bound catecholamines were stable in heparinized or oxalated plasma, whereas there was some loss in citrated plasma. Losses were greatest with EDTA, although the effect was not strictly reproducible. Complete or nearly complete disappearance was found in five out of twenty-one observations, but little or no loss occurred in another six; in the remaining experiments losses varied between 30 and 70%. EDTA sometimes caused losses in the plasma fraction too. Its effects on both fractions were lessened by the exclusion of oxygen [in N_2+CO_2 (95:5, v/v)], but not by the addition of heparin.

With other chelating agents, namely sodium azide (0·005M), potassium cyanide (0·005M), sodium diethyldithiocarbamate (0·001M) and phenylthiourea (0·001M), added either to heparinized or to EDTA plasma (platelet-rich), no clear-cut effects on the stability of catecholamines were observed. On the other hand, the addition of small amounts of adrenaline (2·5 μg./l.) seemed to enhance the disappearance of platelet-bound catecholamines in EDTA plasma, though not in heparinized plasma.

The addition of EDTA decreased the stability of catecholamines in platelets to the same extent, whether the platelets were suspended in plasma, in serum, in serum inactivated at 56° (the latter two fractions were prepared from the same sample of blood as the platelets), in horse serum (Burroughs Wellcome and Co. London), in Ringer solution or in saline. The effect therefore does not depend on an interaction of plasma and platelet factors.

When EDTA was added to a suspension of platelets in Ringer solution or in normal saline, no catecholamines were found in the supernatant after an incubation of 2 hr. Since catecholamines added to saline are stable under these conditions it is unlikely that the losses caused by EDTA are due to leakage. In another experiment platelets which had been incubated in plasma containing EDTA were extracted with acid methanol after they had been passed through alumina. If the incubation had resulted, for instance, in a change of platelet permeability, the removal of catecholamines by the alumina column might have been incomplete and an additional fraction might have been extracted by the more drastic treatment with acid methanol, but, in fact, the extract contained no catecholamines.

Uptake of adrenaline by platelets in vitro

Since it is known that platelets are capable of accumulating serotonin against a concentration gradient (Humphrey & Toh, 1954; Hardisty & Stacey, 1955; Zucker & Borrelli, 1956) their ability to take up adrenaline was investigated. No

Table 7. *Stability of catecholamines in plasma and platelets under various conditions*

Platelet-rich plasma was incubated for 2 hr. at 37° in O_2 or N_2, each containing 5% (v/v) of CO_2. *P* represents volume of anticoagulant solution mixed with 10 vol. of blood.

Expt. no.	Anticoagulant	*P*	Gas	Plasma		Platelets	
				Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
				(% of initial content)			
1	EDTA–thio	3·3	O_2	111	103	0	0
	Heparin	—	O_2	100	87	109	101
2	Heparin	—	O_2	90	103	100	108
	Heparin in EDTA–thio	2·5	O_2	48	50	30	46
	Heparin in EDTA–thio	2·5	N_2	70	70	59	77
3	Sodium EDTA, 2·5% (w/v)	1·7	O_2	24	53	0	0
	EDTA, 2·5% (w/v)	1·7	N_2	92	91	67	69
	Sodium oxalate, 1·3% (w/v)	1·7	O_2	82	92	90	78
	Sodium citrate, 3·8% (w/v)	1·7	O_2	87	100	49	56

Table 8. *Uptake of adrenaline by platelets* in vitro

Blood (8 vol.) was collected in 3·8% (w/v) sodium citrate (1 vol.). Samples (25 ml.) of platelet-rich plasma were incubated at 37° in N_2+CO_2 (95:5, v/v). After 2 hr. the plasma was centrifuged and the residue of platelets washed twice with 10 ml. of 0·9% NaCl. 'Initial' samples were treated immediately. For further details see text.

Expt. no.	Adrenaline added (μg.)	Sample	Platelet-bound fraction: Adrenaline (μg./l. of plasma)	Platelet-bound fraction: Noradrenaline (μg./l. of plasma)	Adrenaline (μmg./10^9 platelets)	Noradrenaline (μmg./10^9 platelets)
1	0	Initial	0·88	2·38	3·06	8·25
	0	Final	0·78	2·28	2·71	7·93
	2	Final	2·64	2·80	9·2	9·7
	4	Final	2·95	2·80	10·3	9·7
2	0	Initial	0·56	1·13	1·35	2·71
		Final	0·39	0·74	0·94	1·77
	5	Initial*	0·43	0·61	—	—
		Final	1·44	1·04	3·44	2·50

* This sample was cooled in ice before the addition of adrenaline, which caused losses of platelets through clumping.

definite uptake was observed after incubation for 1 hr. at 37° in O_2+CO_2 (95:5, v/v), when the concentration of adrenaline in heparinized platelet-rich plasma was increased about tenfold. In similar experiments in which EDTA was used as anticoagulant the addition of adrenaline even potentiated the disappearance of platelet-bound catecholamines. However, when citrate was used as anticoagulant and the concentration of adrenaline was raised by a factor 100–200, unequivocal evidence of adrenaline uptake was obtained (Table 8). In these experiments platelet-rich plasma was incubated in N_2+CO_2 (95:5, v/v) for 2 hr. at 37° and the platelets were washed twice in 0·9% saline before analysis. The efficiency of the washing process was controlled in Expt. 2 of Table 8 by determining the initial value of the platelet-bound fraction after the addition of adrenaline. To minimize the interaction between platelets and adrenaline before analysis the sample of platelet-rich plasma was cooled in ice before the addition of adrenaline; this led to some clumping of platelets and therefore to lower values for platelet-bound catecholamines. The efficiency of the washing procedure, however, has been clearly demonstrated.

The uptake of adrenaline by platelets, though small in relation to the amount of adrenaline added, increased the concentration of platelet-bound adrenaline about threefold. The effect is thus comparable with that observed by Hardisty & Stacey (1955) for serotonin. Born & Hornykiewicz (1957), using still higher concentrations of adrenaline (1 μg./ml.), observed uptake of adrenaline by pig platelets, proportional to the amount of adenosine triphosphate in the platelets.

It has been pointed out above that the concentration gradient [adrenaline in platelets]/[adrenaline in plasma] is about 125. Since [adrenaline in platelets] was increased about threefold, [adrenaline in plasma] would have to be increased by a factor about 400 if the uptake had been due only to diffusion. The concentrations actually used were well below this margin.

Uptake of adrenaline by platelets in vivo

Concentrations of adrenaline such as those found to lead to adrenaline uptake *in vitro* might be reached or exceeded in blood of the suprarenal vein *in vivo*. A procedure which was previously found to lead to an increase of adrenaline in platelet-rich plasma, i.e. convulsion treatment (Weil-Malherbe, 1955), was chosen to study the possible changes of the platelet-bound fraction. Blood samples were obtained from patients undergoing convulsion treatment in a mental hospital. Convulsions were produced by the intravenous injection of Leptazol (5-pentamethylenetetrazol; about 15 mg./kg. body wt.) in four cases and by an electric discharge across the cranium in six cases. One blood sample was withdrawn before the administration of the convulsant agent, a second one immediately after the convulsion and a third one 15 min. later. The results are shown in Table 9. Immediately after the convulsion the adrenaline concentration in platelet-poor plasma rose in all cases and the mean increase was significant. Although there was an increase of the noradrenaline concentration in four cases, the mean increase of noradrenaline was not significant. The adrenaline and noradrenaline contents of the platelets, on the other hand, were significantly decreased in half of the cases, and the mean changes were also significant. This decrease was probably due to an increased adhesiveness of the platelets, since after the convulsion the platelet concentration in plasma was usually decreased, whereas there was

no corresponding change of the platelet count in blood. In these tests relative platelet concentrations were determined in samples of platelet-rich plasma by a turbidity method (Cruz, 1954) and figures for the catecholamine content of absolute numbers of platelets are not available, but this aspect will be investigated further.

Fifteen minutes after the convulsion the 'plasma' fraction of adrenaline was still significantly increased. Other increases were not statistically significant. There was thus no evidence for an uptake of catecholamines *in vivo* by platelets under these conditions, and it appears that the increase of adrenaline concentration previously observed is confined to the fraction present in platelet-poor plasma.

The concentration of catecholamines in bovine plasma obtained from the slaughterhouse is higher than in human plasma; this has been attributed to a discharge of catecholamines at the time of blood collection (Weil-Malherbe & Bone, 1957*b*). The proportion of platelet-bound catecholamines in bovine plasma was therefore studied in a small series of experiments (Table 10). The sum of free plus platelet-bound fractions is almost identical with the mean concentrations of adrenaline and noradrenaline previously found in unfractionated ox plasma (Weil-Malherbe & Bone, 1957*b*). The platelet-bound proportion is a little lower than in human plasma, but the difference is not significant. On the other hand, the content per unit number of platelets is about five times higher than in human plasma. The adrenaline content of pig platelets is higher still, according to Born & Hornykiewicz (1957). Whether this difference between human platelets on the one hand and bovine or pig platelets on the other is due to uptake of pre-mortally discharged catecholamines by the animal platelets or to species differences, remains to be investigated. Considerable species differences are known to exist in histamine and serotonin contents of platelets (Humphrey & Jaques, 1954).

DISCUSSION

The partial association of circulating catecholamines with blood platelets brings to mind the fact that tissue catecholamines are also partly bound to particles. This has been demonstrated for the adrenal medulla (Hillarp & Nilson, 1954; Blaschko, Hagen & Welch, 1955), phaeochromocytoma (Burger & Langemann, 1956), spleen and sympathetic nerves (Euler & Hillarp, 1956) and brain (Weil-Malherbe & Bone, 1957*c*). It seems natural to assume that there is an inherent functional purpose in this arrangement which may serve to prevent the reaction of catecholamines with receptor groups and inactivating enzymes. The particle-bound fraction may thus be conceived as an operational reserve from which active amines are released as required. Although this concept is well founded for the particles of adrenal medulla,

Table 9. *Effect of convulsion treatment on the concentration of catecholamines in plasma and platelets*

Sample 1: blood was withdrawn before treatment; sample 2: blood was withdrawn immediately after convulsion; sample 3: blood was withdrawn 15 min. after convulsion. Heparin was used as anticoagulant. Values are means ± S.E.M. from ten cases. For details see text. A = Adrenaline; N = noradrenaline.

Sample no.	Plasma A	Plasma N	Platelets A	Platelets N	10^{-3} × Platelet count/μl. of whole blood	$E_{620\,m\mu}$ of platelet-rich plasma (1:20)
	(μg./l. of plasma)					
1	1·13 ± 0·15	3·79 ± 0·36	1·32 ± 0·16	4·53 ± 0·47	227 ± 29	0·176 ± 0·028
2	2·15 ± 0·41	4·98 ± 0·99	1·08 ± 0·15	3·93 ± 0·54	250 ± 32	0·116 ± 0·025
3	1·55 ± 0·25	4·19 ± 0·53	1·43 ± 0·14	4·74 ± 0·45	279 ± 36	0·173 ± 0·025
	Percentage change after convulsion (value before convulsion = 100)					
2	191·2 ± 28·9*	131·8 ± 29·9	82·0 ± 5·5*	86·8 ± 5·4†	110·1 ± 9·3	66·0 ± 12·2†
3	136·9 ± 15·2†	110·5 ± 15·1	108·4 ± 8·9	104·6 ± 11·8	122·7 ± 6·0*	98·4 ± 8·6

* Significantly different from 100 ($P \leqq 0{\cdot}01$). † Significantly different from 100 ($P < 0{\cdot}05 > 0{\cdot}01$).

Table 10. *Catecholamine distribution in bovine plasma*

Blood was collected at the slaughterhouse with heparin as anticoagulant. Values are means of four experiments ± S.E.M.

	Plasma (μg./l.)	Platelets (μg./l. of platelet-rich plasma)	μmg./10^9 platelets*	Percentage in platelets of total adrenaline and noradrenaline
Adrenaline	4·0 ± 0·72	1·65 ± 0·27	9·8	30·8 ± 6·9
Noradrenaline	8·62 ± 2·19	6·15 ± 1·51	33·5	43·1 ± 11·9

* Means of two experiments.

its applicability to blood platelets remains to be established.

It seems that catecholamines are less easily detached from platelets than is serotonin, as they do not pass into solution during clotting and do so only partly after lysis by freezing or exposure to a surface-active agent. Also, any changes in the platelets which may be produced by contact with glass do not substantially affect the retention of catecholamines; this, at least, is found when the platelets are suspended in a medium containing EDTA.

The unfavourable effect of EDTA on the stability of platelet-bound catecholamines *in vitro* requires some comment. The underlying reaction is probably an oxidation, since the destruction is smaller in nitrogen. It is well known that EDTA activates iron-catalysed autoxidations. Green, Mazur & Shorr (1956) have shown that EDTA accelerates about tenfold the oxidation of adrenaline by ferritin or Fe^{3+} ions at pH 7. Citrate has no effect on this reaction and oxalate inhibits it. The same order of activity was observed in our experiments on the effects of these anticoagulants on the stability of platelet-bound catecholamines. It seems likely therefore that EDTA acts by activating an iron-catalysed oxidation. Whether the catalyst is ferritin is doubtful since ferritin has not yet been demonstrated in blood except in certain pathological conditions (Granick, 1951). The catalyst probably occurs at a higher concentration in platelets where the effect is stronger than in plasma.

It might be concluded that the use of EDTA as anticoagulant in adrenaline estimations is inadvisable and that it ought to be replaced by heparin, citrate or oxalate. However, none of these equals EDTA in its ability to prevent adhesion and clumping of platelets (Dillard, Brecher & Cronkite, 1951). Moreover, the instability of catecholamines in platelets suspended in EDTA (Table 4) was observed in experiments at 37° and lasting for 2 hr. The yield of platelets is therefore highest with EDTA; slight losses of platelet catecholamines during processing cannot be ruled out, but they can be kept to a minimum by avoiding delay and by cooling of the sample.

Leach & Heath (1956) suggested that adrenaline is oxidized in plasma by coeruloplasmin, a copper-containing protein. In their experiments adrenaline was added to plasma *in vitro* at a final concentration about 10^6 times the physiological concentration. We have added diethyldithiocarbamate or phenylthiourea, both powerful inhibitors of catalysis by copper, to heparinized platelet-rich plasma and found that they did not affect the stability of preformed catecholamines in either platelets or plasma. We conclude therefore that there is no significant copper-catalysed oxidation under these conditions. It is probable that normal protective mechanisms are swamped when unphysiologically high concentrations are used and the physiological significance of the observations of Leach & Heath is therefore doubtful.

SUMMARY

1. The distribution of adrenaline and noradrenaline between plasma and blood platelets has been studied by a fluorimetric method.

2. In 116 samples of human plasma the platelets contained, on the average, a little over 50% of the total catecholamines present in platelet-rich plasma. The platelet-bound proportion of adrenaline correlated with the platelet-bound proportion of noradrenaline. The mean amounts contained in 10^9 platelets were 2 μmg. of adrenaline and 7 μmg. of noradrenaline. It was calculated that the concentration of catecholamines is about 125 times higher in platelets than in plasma.

3. The distribution of catecholamines between platelets and plasma was unaffected by contact with uncoated glass surfaces. Some anticoagulants decreased the yield of platelets in platelet-rich plasma by causing clumping and adhesion. Highest platelet yields were obtained with ethylenediaminetetra-acetate.

4. No catecholamines passed into serum from platelets during clotting, whether spontaneous or induced. Lysis of platelets by freezing and thawing or by treatment with a surface-active agent resulted in a partial release. Extraction with butanol was practically quantitative.

5. Ethylenediaminetetra-acetate increased the disappearance in oxygen of platelet-bound catecholamines *in vitro*.

6. No uptake of adrenaline by platelets was observed in heparinized platelet-rich plasma at an adrenaline concentration of about 10 μg./l. An uptake resulting in a final concentration of platelet-bound adrenaline of about three times the initial concentration was found in citrated platelet-rich plasma at an adrenaline concentration of 80–200 μg./l.

7. In confirmation of earlier results an increase of adrenaline was found in platelet-rich plasma after convulsion treatment. This increase was confined to the 'free' fraction.

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Spectral Methods of Characterizing Anthocyanins

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Because of the close relationship that exists between anthocyanins and genetic factors controlling flower colour in higher plants (Lawrence, 1950), considerable efforts have been made to devise rapid and simple methods of characterizing this group of glycosidic pigments. Existing methods, e.g. the well-known colour and distribution tests (Robinson & Robinson, 1932), although of value, provide only limited information about the glycosidic nature of the anthocyanins. More recently paper-partition chromatography has been widely used as an alternative to the older tests (Bate-Smith, 1948, 1949; Hayashi & Abe, 1956), and it provides an excellent method of obtaining pure pigments on a small scale. A method of identifying the sugars of anthocyanins with this technique has recently been described (Harborne & Sherratt, 1957*a*, *b*). In a study of the genetic control of anthocyanin production in a number of different plants (Harborne, 1958*a*), the absorption spectra of a large number of chromatographically pure pigments were measured. As a result, new ways in which spectral measurement can be used for characterizing anthocyanins became apparent.

Although the spectra of many anthocyanins have been examined in the past (Schou, 1927; Hayashi, 1936; Robinson, 1954), little attempt was made to apply these data to the characterization of these pigments. More recently, spectral measurements have been used for identifying anthocyanidins, mainly in connexion with studies of plant leucoanthocyanins (Bate-Smith, 1954; Roux, 1957*a*, *b*). In addition, the effect of aluminium chloride on the spectra of anthocyanins has been described as distinguishing pigments containing *o*-dihydroxyl groups (Geissman, Jorgensen & Harborne, 1953; Geissman & Jurd, 1955).

In the present work complete spectral data are given for all those anthocyanidins which occur naturally as glycosides, as distinct from those which are obtained by treatment of leucoanthocyanins with acid. A method of distinguishing the two main classes of anthocyanin, the 3- and the 3:5-glycosides, is described. Some anthocyanins occur naturally in association with hydroxy aromatic acids in acylated form and the nature and amount of acyl components have now been determined by spectral means.

MATERIALS AND METHODS

Sources of pigments

Anthocyanidins. Small crystalline samples of authentic delphinidin, cyanidin, petunidin, malvidin and hirsutidin were available. Larger amounts of these pigments were prepared from suitable plant material. Pelargonidin was

obtained by acid hydrolysis under standard conditions (Geissman, 1955) of petal extracts of *Dianthus caryophyllus* var. William Sim. Similarly, peonidin was obtained from *Peonia* spp., and rosinidin from *Primula rosea* and *P. polyanthus*. Luteolinidin, apigeninidin and peonidin, and their 5-*o*-benzoyl derivatives, were synthesized by known methods (Murakami & Robinson, 1928; León & Robinson, 1931; Robinson, Robinson & Todd, 1934).

Simple anthocyanins. The following pigments were either available in crystalline form, or isolated in crystalline form, from authentic plant sources (Wawzonek, 1951): pelargonidin 3-monoglucoside and 3:5-diglucoside, cyanidin 3-rhamnoglucoside, 3-gentiobioside and 3:5-diglucoside, peonidin 3-monoglucoside and 3:5-diglucoside, delphinidin 3-monoglucoside and 3:5-diglucoside, petunidin 3-monoglucoside, malvidin 3-monoglucoside and 3:5-diglucoside.

The following pigments, which have not been fully characterized before, were obtained from the plant sources given in parentheses. Pelargonidin derivatives: the 3-rhamnoglucoside (orange–yellow and pink flowers of *Antirrhinum majus*, dark-red tubers of *Solanum phureja*), the 3-gentiobioside (flowers of *Papaver rhoeas*, *P. orientale*, nasturtium), the 3-diglucosido-7 (or -4′)-glucoside (flowers of *Papaver orientale*), the 3-rhamnoglucosido-5-glucoside (red tubers of *Solanum phureja*) and the 3-diglucosido-5-glucoside (red skin of *Raphanus sativum*). Cyanidin derivatives: the 3-monoglucoside and the 3-xyloglucoside (berries of *Sambucus nigra*, stems and leaves of *Streptocarpus* spp.). Peonidin 3-rhamnoglucosido-5-glucoside (red-and-blue tubers of *S. phureja*). Delphinidin 3-rhamnoglucoside (deep-blue tubers of *S. phureja*). Petunidin and malvidin 3-rhamnoglucosido-5-glucoside (purple tubers of *S. andigenum* var. Congo). When the pigments occur in acylated form (see below), the simple anthocyanins were obtained by deacylation of the complex with alkali (Robinson & Robinson, 1932).

The following pigments were obtained by partial acid hydrolysis of certain anthocyanins (Harborne & Sherratt, 1957*b*), and were separated from the other products of partial hydrolysis by paper chromatography. The 5-glucosides of pelargonidin and peonidin were obtained from the corresponding 3:5-diglucosides. Petunidin 3:5-diglucoside was similarly obtained from the corresponding 3-rhamnoglucosido-5-glucoside.

Acylated anthocyanins. Monardein and salvianin were isolated from flowers of *Monarda didyma* and *Salvia splendens* respectively (Karrer & Widmer, 1928; Willstätter & Bolton, 1917). Raphanin was isolated from the skin of the radish (*Raphanus sativum*). Pelanin, petanin and negretein were obtained from the tubers of cultivated potatoes (*S. phureja* and *S. andigenum* var. Congo).

Methods

Isolation. Well-characterized pigments were isolated by the usual methods (Wawzonek, 1951). Details of the isolation of the previously uncharacterized pigments will be given in later papers. The general method of isolating anthocyanins by paper chromatography is a modification of that used by other authors (e.g. Nordström, 1956).

Freshly collected plant material was extracted by maceration with methanol–conc. HCl (97:3, v/v) at room temperature. The extract was centrifuged, and filtered through Celite, and concentrated to a small volume *in vacuo* at 50–60° and refiltered. The concentrated extract was applied as a thin band near one edge of several sheets of Whatman no. 3 filter paper. When dry, the papers were equilibrated for 24 hr. in a tank with the lower phase of a mixture of butan-1-ol–2N-HCl (1:1, v/v) and then developed with the upper phase of the same mixture.

The anthocyanin bands were cut out from the dried chromatograms and eluted separately with water–methanol–acetic acid (25:70:5, by vol.). The individual bands were then separated from the polyphenolic impurities by application on Whatman no. 3 paper and development with butan-1-ol–acetic acid–water (4:1:5, by vol., upper phase) and by repeating the process with water–acetic acid (85:15, v/v) as the solvent.

Purification. Anthocyanidins were purified by chromatography on Whatman no. 1 paper by development with acetic acid–conc. HCl–water (30:3:10, by vol.) or formic acid–conc. HCl–water (5:2:3, by vol.).

Anthocyanin specimens were purified by chromatography on Whatman no. 3 paper by development with one or more of the solvent systems: butan-1-ol–acetic acid–water (4:1:5, by vol., upper phase), butan-1-ol–2N-HCl (1:1, v/v, upper phase), acetic acid–water–conc. HCl (15:82:3, by vol.) and conc. HCl–water (3:97, v/v). The anthocyanins were eluted from the paper with water–methanol–acetic acid (25:70:5, by vol.) and the eluates were concentrated *in vacuo* at 50°. After the final purification, the eluate containing the anthocyanin was evaporated to dryness *in vacuo*. The anthocyanin thus obtained was assumed to be pure if it (*a*) gave a single anthocyanidin after acid hydrolysis; (*b*) appeared as a single spot on paper chromatograms developed with different solvent mixtures; (*c*) had a characteristic absorption spectrum in the region 250–600 mμ.

Spectrophotometry. All measurements were made with a Unicam SP. 500 spectrophotometer. The pure dry pigments were dissolved in methanol containing 0·01% of conc. HCl and the solutions diluted to give an optical-density reading in the range 0·800–1·300 at the visible maxima. For measurements in the ultraviolet region, the solutions of those anthocyanins obtained from eluting chromatograms were measured against a solution obtained from an appropriate blank area of the chromatogram, prepared at the same time as the corresponding pigment solution.

For the purpose of measuring spectral shifts in the presence of aluminium chloride, three drops of a solution of the anhydrous salt in ethanol (5%, w/v) were added to the cell solution. Measurements of the shift were made as quickly as possible, since in some cases the colour of the resulting solution faded on standing.

For measuring the spectra of mixtures of pelargonidin 3:5-diglucoside and *p*-coumaric, caffeic and ferulic acids, 1 m-mole of each compound in pure, anhydrous form was dissolved in 50 ml. of methanol containing 0·01% of conc. HCl. Portions (1 ml.) of these stock solutions were mixed together in varying proportions and the solution was made up to 10 ml. The compounds were previously purified by recrystallization and then dried *in vacuo* at 100° over phosphorus pentoxide.

RESULTS

The absorption maxima of some 50 anthocyanidins and anthocyanins in the visible region have been measured and the results are collected in Tables 1–3.

No attempt has been made to record values of the extinction coefficients, since many compounds were not available in sufficient amounts for accurate weighing. All pigments were carefully purified by paper chromatography, as described in the Materials and Methods section.

The same solvent (methanol containing 0·01 % of hydrochloric acid) was always used, in order to avoid shifts in the position of the visible maxima due to pH changes (Sondheimer & Kertesz, 1948) or solvent effects. Many spectra were also recorded in ethanol and, less frequently, in water, both containing 0·01 % of hydrochloric acid. In general, the values for $\lambda_{max.}$ in ethanol are about 10 mμ towards longer wavelengths and in water about 15 mμ towards shorter wavelengths than those given in methanol. For example, the values for cyanidin 3-rhamnoglucoside are 507 mμ in water, 523 mμ in methanol and 533 mμ in ethanol.

Spectra were also measured in the presence of traces of aluminium chloride at a suitable pH (Geissman & Jurd, 1955) and the resulting spectral shifts of pigments containing two or more adjacent hydroxyl groups (Geissman *et al.* 1953) noted. The results obtained here with a much larger number of anthocyanins confirm the use of this reagent for detecting the presence of *o*-dihydroxyl groups in these pigments.

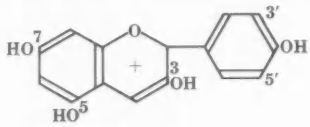
Spectra of the naturally occurring anthocyanidins

The visible spectra of all the anthocyanidins known to occur in nature as glycosides are collected in Table 1. Their structures are given in Fig. 1. Six of these pigments are of frequent occurrence, namely pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Two others, hirsutidin (7-methylmalvidin) and rosinidin (probably 7-methylpeonidin) have been found only in a few plants (Karrer & Widmer, 1927; Harborne, 1958*b*; Forsyth & Simmonds, 1957). The other two, which lack a 3-hydroxyl group, namely apigeninidin (Robinson *et al.* 1934) and luteolinidin (Harborne, 1957) also occur rarely. The value of spectral measurements for distinguishing them is clear, and the method fails only with delphinidin and petunidin. Fortunately, these two anthocyanidins have quite different chromatographic properties (Bate-Smith, 1954). Apigeninidin and luteolinidin are exceptional in also giving stable spectral maxima in methanolic alkali, with bathochromic shifts of 56 and 70 mμ respectively.

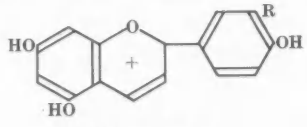
As a result of using two different solvents, methanol and ethanol, it is apparent that there are small but detectable differences between the visible maxima of cyanidin and peonidin, and between delphinidin, petunidin and malvidin. This point has not been noted previously (cf. Bate-Smith, 1954). Whereas methylation of the 3′- and 5′-hydroxyl groups causes only small hypsochromic

Table 1. *Spectra and characteristics of the naturally occurring anthocyanidins in the visible region*

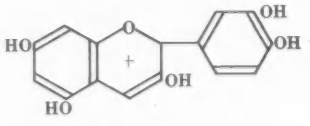
Pigment	Spectral maxima $\lambda_{max.}$ (mμ) Methanol–HCl	Ethanol–HCl	$AlCl_3$ shift $\Delta\lambda$ (mμ)
Hirsutidin	536	545	0
Malvidin	542	554	0
Petunidin	543	558	24
Delphinidin	546	557	23
Rosinidin	524	534	0
Peonidin	532	542	0
Cyanidin	535	545	18
Pelargonidin	520	530	0
Luteolinidin	493	503	52
Apigeninidin	476	483	0



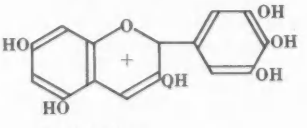
Pelargonidin



Apigeninidin (R=H)
Luteolinidin (R=OH)



Cyanidin
Peonidin (3′-methyl ether)
Rosinidin (7:3′-dimethyl ether?)



Delphinidin
Petunidin (3′-methyl ether)
Malvidin (3′:5′-dimethyl ether)
Hirsutidin (7:3′:5′-trimethyl ether)

Fig. 1. Naturally occurring anthocyanidins.

shifts, a more substantial shift of 8–10 mμ is produced when the 7-hydroxyl group is methylated (rosinidin, hirsutidin).

Characterization of the anthocyanins

The spectra of some 30 anthocyanins have been measured in the range 240–600 mμ. All the simple anthocyanins show similar absorption in the ultraviolet region and it is only in the visible spectra that differences in absorption are apparent. The position of the visible maxima, and the percentage intensity of absorption at 440 mμ compared with that at the maximum wavelength (i.e. between 500 and 550 mμ), have been measured and the results are collected in Table 2, together with similar data for the related anthocyanidins.

There are two ways in which these measurements can be used to characterize anthocyanins. First,

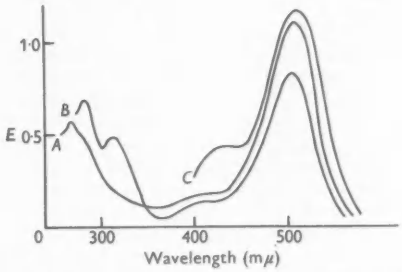


Fig. 2. Absorption spectra: curve *A*, pelargonidin 3:5-diglucoside; curve *B*, pelargonidin 3:5 diglucoside acylated with *p*-coumaric acid (monardein); curve *C*, pelargonidin 3-monoglucoside. The concentrations of the pigments were about 50 μM, in methanolic 0·01% (w/v) HCl.

Table 2. *Spectral maxima of anthocyanins in the visible region*

	Pigment*	$\lambda_{max.}$† (mμ)	$E_{440}/E_{max.}$‡ (as %)	Mean (%)
Pelargonidin and derivatives	With 5-hydroxyl group free			
	Pelargonidin	520	39	
	Pelargonidin 3-monoglucoside	506	38	
	Pelargonidin 3-rhamnoglucoside	508	40	39
	Pelargonidin 3-gentiobioside	506	36	
	Pelargonidin 3-diglucosido-7 (or 4′-)-glucoside	498	42	
Cyanidin and derivatives	Cyanidin	535§	19	
	Cyanidin 3-monoglucoside	525§	22	
	Cyanidin 3-rhamnoglucoside	523§	23	
	Cyanidin 3-gentiobioside	523§	25	23
	Cyanidin 3-xyloglucoside	523§	22	
	Peonidin	532	25	
	Peonidin 3-monoglucoside	523	26	
Delphinidin and derivatives	Delphinidin	544§	16	
	Delphinidin 3-monoglucoside	535§	18	
	Delphinidin 3-rhamnoglucoside	537§	17	
	Petunidin	543§	17	18
	Petunidin 3-monoglucoside	535§	18	
	Malvidin	542	19	
	Malvidin 3-monoglucoside	535	18	
Pelargonidin and derivatives	With 5-*O*-substituent			
	Pelargonidin 5-glucoside	513	15	
	Pelargonidin 3:5-diglucoside	504	21	
	Pelargonidin 3-rhamnoglucosido-5-glucoside	505	19	20
	Pelargonidin 3-diglucosido-5-glucoside	503	21	
	Monardein	505	21	
	Salvianin	505	20	
Cyanidin and derivatives	Cyanidin 3:5-diglucoside	522§	13	
	Peonidin 3:5-diglucoside	523	13	
	Peonidin 3-rhamnoglucosido-5-glucoside	523	12	12
	Peonidin 5-glucoside	528	12	
	Peonidin 5-benzoate	528	11	
Delphinidin and derivatives	Delphinidin 3:5-diglucoside	534§	11	
	Petunidin 3:5-diglucoside	533§	10	
	Petunidin 3-rhamnoglucosido-5-glucoside	535§	10	10
	Malvidin 3:5-diglucoside	533	12	
	Malvidin 3-rhamnoglucosido-5-glucoside	534	9	
	Negretein	536	9	

* As the chloride.
† Measured in methanolic 0·01% HCl; values are correct to ±1 mμ.
‡ Average of five determinations, mean error ±2%.
§ Only these pigments had their spectra shifted to the blue region by the addition of ethanolic $AlCl_3$.

the spectral characteristics of a particular anthocyanin are obviously related to the anthocyanidin from which it is derived. The three main groups, those based on pelargonidin, cyanidin and delphinidin, are well differentiated spectrally, a reflexion of their characteristically different colours in solution and on chromatograms. Furthermore, cyanidin, delphinidin and petunidin glycosides can be distinguished from the other anthocyanins by the use of aluminium chloride (see above).

Secondly, spectral measurements are of value in determining the position of attachment of the sugars in the anthocyanin molecule. The general effect of glycosidation on the visible spectrum of the anthocyanidin molecule (represented by pelargonidin-3:5:7:4′-tetrahydroxyflavylium) is to shift it towards shorter wavelengths. The amount of this hypsochromic shift depends upon which and how many hydroxyl groups are glycosidated. The largest shift is caused by the introduction of a sugar residue in the 3-position. Thus pelargonidin has $\lambda_{max.}$ at 520 mμ, its 3-glycosides at 505 mμ ($\Delta\lambda = 15$ mμ); cyanidin has $\lambda_{max.}$ 535 mμ, its 3-glycosides at 523 mμ ($\Delta\lambda = 12$ mμ); and delphinidin has $\lambda_{max.}$ 544 mμ, its 3-glycosides at 534 mμ ($\Delta\lambda = 10$ mμ). The hypsochromic shift caused by the introduction of a sugar residue in the 5-position is 7 mμ (cf. pelargonidin, peonidin and their 5-glucosides, Table 2). Only very small differences in the spectral maxima can be detected between anthocyanins containing sugars in both the 3- and 5-positions and 3-glycosides. However, it has now been observed that the absorption spectra in the 400–570 mμ region of all anthocyanidins with sugar or benzoyl residues on the 5-hydroxyl group show characteristic differences from those of anthocyanidins in which the 5-hydroxyl group is free. The spectra of the latter group all show a distinct shoulder to the main absorption peak in the 410–450 mμ region (compare curves *A* and *C*, Fig. 2). The best way of recording the differences is to compare the ratio of the optical density at an arbitrarily chosen wavelength (i.e. 440 mμ) with that at the wavelength of maximal absorption. It will be seen (Table 2) that within the three main groups of pigment, the anthocyanins fall into two classes depending on whether or not they have a free 5-hydroxyl group. In effect, the percentage intensity at 440 mμ of 5-substituted anthocyanidin is approximately half that of the corresponding anthocyanidin in which the 5-hydroxyl group is free. This relationship also holds true in the apigeninidin and luteolinidin series (J. B. Harborne, unpublished work). This provides a valuable new means of distinguishing between the two main classes of anthocyanin, the 3- and the 3:5-glycosides.

Acylated anthocyanins

The absorption spectra of some acylated anthocyanins are given in Table 3. The pigments examined are typical of the majority of acylated anthocyanins that have been examined to date, as they contain a hydroxy aromatic acid as their acyl component. They include several pigments of the cultivated potato, all of which contain *p*-coumaric acid (*p*-hydroxycinnamic acid) (Chmielewska, 1936; Harborne, 1956). Salvianin and

Table 3. *Absorption spectra of acylated anthocyanins and pelargonidin 3:5-diglucoside–cinnamic acid mixtures*

Acylated anthocyanins			$\lambda_{max.}$ in methanol–HCl (mμ)	$E_{max.}$ acid/ $E_{max.}$ pigment (as %)
Pigment	Aglycone	Acyl group		
Pelanin	Pelargonidin	*p*-Coumaric acid	289, 313, 505	67
Monardein	Pelargonidin	*p*-Coumaric acid	286, 313, 507	60
Salvianin	Pelargonidin	Caffeic acid	285, 329, 507	48
Raphanin A	Pelargonidin	*p*-Coumaric acid	286, 313, 505	60
Raphanin B	Pelargonidin	Ferulic acid	282, 328, 505	52
Petanin	Petunidin	*p*-Coumaric acid	282, 308, 536	66
Negretein	Malvidin	*p*-Coumaric acid	282, 308, 535	71

Cinnamic acids with pelargonidin 3:5-diglucoside	Ratio	$\lambda_{max.}$ in methanol–HCl (mμ)	$E_{max.}$ acid/ $E_{max.}$ pigment (as %)
Alone	—	269, —, 505	6
p-Coumaric acid	1:1		57
	1:2	288, 312, 505	107
	2:1		31
Caffeic acid	1:1		47
	1:2	287, 328, 505	83
Ferulic acid	1:1		49
	1:2	287, 326, 505	91

monardein, two well-characterized pigments, have also been examined. In addition, a new acylated pelargonidin glycoside from the radish has been looked at.

Confirmation of the presence (or absence) of a hydroxy aromatic acid as an acyl component can be made by spectral means, as has already been indicated (Harborne, 1956). The spectra of anthocyanins acylated with *p*-coumaric acid (curve *B*, Fig. 2) show two peaks in the ultraviolet at 289 and 310 mμ, due to the superimposition of the absorption of the cinnamic acid ($\lambda_{max.}$ 310 mμ) upon that of the pigment absorption. The spectra of simple anthocyanins (curve *A*, Fig. 2) exhibit a simple peak at about 270 mμ and only very weak absorption at 310 mμ. In addition, the ratio of the intensity at 310 mμ to that at the colour maximum (say, 505 mμ) is a measure of the molar ratio of cinnamic acid to anthocyanin in the complex pigment. The results of this estimation are in Table 3, which also includes values for synthetic mixtures, in varying proportions, of pelargonidin 3:5-diglucoside with *p*-coumaric, caffeic (3:4-dihydroxycinnamic) and ferulic (3-methoxy-4-hydroxycinnamic) acid. The value for monardein, known to be pelargonidin 3:5-diglucoside acylated with one molecule of *p*-coumaric acid (Karrer & Widmer, 1928), agrees well with the value for a synthetic mixture. In addition, the results show that the potato pigments (pelanin, petanin and negretein) all have the same molar ratio of anthocyanin to *p*-coumaric acid of 1:1. In addition, the spectral ratio appears to be independent of the nature of the anthocyanin, since pelargonidin and malvidin derivatives (e.g. pelanin and negretein) give values of the same order.

This ratio has been measured in anthocyanins acylated with other cinnamic acids, and the results agree with synthetic mixtures of pelargonidin 3:5-diglucoside with the respective acid. For example, salvianin, thought to contain *p*-coumaric acid by Karrer & Widmer (1929), is a derivative of caffeic acid and has its spectral maximum in the ultraviolet at 329 mμ (caffeic acid $\lambda_{max.}$ 330 mμ) and not at 310 mμ. A preliminary examination of raphanin, the pigment of the radish, showed it to be an acylated pelargonidin 3-diglucosido-5-glucoside (Harborne & Sherratt, 1957*b*). It has now been found that the pigment contains two components, raphanin A, which is acylated with one molecule of *p*-coumaric acid, and raphanin B, which is acylated with one molecule of ferulic acid (see Table 3).

In all cases, the cinnamic acids present in the above pigments and obtained from them by alkaline hydrolysis have been identified by spectral and chromatographic comparison with authentic compounds.

DISCUSSION

In searching for new methods of characterizing anthocyanins, it should be remembered that they are difficult compounds to deal with by the usual techniques of organic chemistry. In solution, they are unstable to light and pH changes. They are difficult to isolate in a pure state as a general rule. Many of them do not have sharp melting points and do not give meaningful results on elementary analysis. No suitable derivatives are known for characterizing them. The procedure of methylation and hydrolysis, commonly used with flavones for determining the position of sugar residues, is of limited value in the anthocyanin series (Nordström, 1956).

The present study provides additional means for characterizing anthocyanins, which avoid the above difficulties. Spectral methods require only small amounts of pigment and are therefore ideally suited for biochemical studies. The examination of the spectra and chromatographic behaviour of anthocyanins provides the basic information obtainable by the Robinson tests and, in addition, certain other structural conclusions can be drawn. With the naturally occurring anthocyanidins, spectral and chromatographic methods have already been used in a number of investigations (e.g. Dodds & Long, 1955) for identification purposes. Data for the less-common anthocyanidins have not generally been available until now. That these pigments have quite different spectral properties from the commonly occurring compounds strengthens the value of this approach to identification of anthocyanidins.

The most valuable feature of the present study is that the position of the sugar residues in the anthocyanin molecule may be deduced from the spectral data. This is particularly clear in comparing 3- with 3:5-glycosides. It is also likely that 3:7-glycosides are spectrally distinguishable from 3- or 3:5-glycosides. That this is true, at least in the pelargonidin series, is borne out by the discovery of a novel pelargonidin glycoside in the petals of *Papaver orientale*, which has $\lambda_{max.}$ 498 mμ, as compared with $\lambda_{max.}$ 505 mμ of pelargonidin 3- and 3:5-glycosides. A preliminary investigation of this pigment (Harborne & Sherratt, 1957*b*) indicates that it has two sugar residues in the 3-position, none in the 5-position and one in the 7- or 4′-position. Of the two alternative structures, that in which the third sugar is in the 7-position is the more likely for the following reason. 4′-Glycosides are quite different from other glycosides in their colour reactions in alkaline media (León, Robertson, Robinson & Seshadri, 1931), and no such difference has been noted with this pigment.

Finally, it should be pointed out that these methods have successfully been applied to studies of the anthocyanins present in colour mutants of *Solanum phureja* and *Primula sinensis*, and in a variety of other plant material. Provisional structures for a number of newly discovered pigments have been advanced on the basis of spectral and chromatographic data, of identifying the sugars present (Harborne & Sherratt, 1957*a*) and of examining the simpler glycosides produced by partial acid hydrolysis. Further details of these results will appear in later papers.

SUMMARY

1. Spectral data for both the common and rare naturally occurring anthocyanidins are given.

2. Spectral measurements have been made of a large number of anthocyanins. It is shown that 3- and 3:5-glycosides have characteristically different absorption properties in the 400–450 mμ region.

3. Measurements of the absorption spectra in the ultraviolet region of anthocyanins acylated with hydroxy aromatic acids can be used to determine the hydroxy aromatic acid:pigment ratio in these complex pigments.

4. The value of these measurements for characterizing anthocyanins is discussed.

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A Sensitive Method for Estimation of Oxaloacetate

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The coupling of acetoacetate with an excess of diazotized *p*-nitroaniline has been utilized by Walker (1954) to develop a sensitive colorimetric method for the estimation of acetoacetate. With defined conditions at pH 4·9–5·0, the reaction proceeds as in equation 1 (R = *p*-NO_2.C_6H_4) with the formation of *NN'*-bis-(*p*-nitrophenyl)-*C*-acetylformazan.

$$CH_3 \cdot CO \cdot CH_2 \cdot CO_2H + 2RN_2 \cdot OH \rightarrow CH_3 \cdot CO \cdot C(N{:}NR)(N \cdot NHR) + CO_2 + 2H_2O. \quad (1)$$

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In the present investigation, the method of Walker (1954) has been modified to measure very small quantities (0·003–0·03 μmole) of oxaloacetic acid in the presence of moderate concentrations (0·1 μmole) of acetoacetate. The method has been applied to the estimation of oxaloacetate in the livers of both fed and fasted rats.

EXPERIMENTAL AND RESULTS

Materials

p-*Nitroaniline* (0·05%, *w/v*) *in* 0·05N-*hydrochloric acid.* This solution was prepared by dissolving the *p*-nitroaniline (recrystallized from hot water) in boiling water and the required amount of HCl.

Diazo reagent. This was prepared immediately before use as described by Walker (1954). A volume (3 ml.) of $NaNO_2$ solution (0·5%, w/v; prepared daily) was added to 20 ml. of cold *p*-nitroaniline solution. The colourless solution was kept in an ice bath and 7 ml. of 0·2N-sodium acetate was added just before use.

Acetoacetic acid. This was provided through the generosity of Mr R. Kulka and had been prepared according to the method of Krebs & Eggleston (1945).

Oxaloacetic acid. This was provided through the generosity of Mr R. Hems and had been prepared according to the method of Wohl & Oesterlin (1901) and Wohl & Claussner (1907).

Tissue extracts. Tissue homogenates (20%) in a final concentration of 3% (w/v) trichloroacetic acid were prepared with livers of normal male rats [4 ml. of 5% (w/v) glucose was injected intraperitoneally 15 min. before death] and with livers of male animals fasted for either 24 or 69 hr. After decapitation of the rat, the liver was removed as rapidly as possible and plunged into liquid oxygen. The frozen liver was then weighed, crushed at $-10°$ in a Hughes press (Hughes, 1951), rapidly transferred to a stainless-steel homogenizer containing 4 vol. of cold 3·75% (w/v) trichloroacetic acid and homogenized at 0–5°. A sample was taken for dry-weight determination (2 ml. contained approx. 100 mg.). The rest of the homogenate was centrifuged at approx. 900 *g* for 5 min. and a portion of the supernatant taken for analysis.

Standard method

The method finally adopted is a direct modification of that of Walker (1954). The modifications are based upon the findings that (1) the compound produced by the reaction of an excess of diazo reagent with oxaloacetate is unstable in acid (Walker, 1954), whereas that formed with acetoacetate is stable, and that (2) the addition of $NaNO_2$ at acid pH removes ascorbic acid which otherwise would interfere with the estimation of both oxaloacetate and acetoacetate. Measurements of the colour of solutions A, B and C treated as described below permit the simultaneous estimation of oxaloacetic and acetoacetic acids in biological materials.

To each of two glass-stoppered test tubes containing 2 ml. of supernatant from a 20% tissue homogenate (solutions A and B) is added 0·4 ml. of 0·25% $NaNO_2$ (to remove ascorbic acid). Exactly 30 sec. later 2 ml. of M-sodium acetate–acetic acid buffer, pH 5·2, is added, followed immediately by 3 ml. of freshly prepared cold diazo reagent. After incubation for 20 min. at 25°, the tubes are placed in an ice-bath for 3 min. The colour due to reaction of the diazo reagent with both acetoacetate and oxaloacetate is determined with solution A by adding 6 ml. of ethyl acetate and then 6 ml. of 5N-HCl. The colour is immediately and quantitatively extracted into ethyl acetate by shaking the tubes 30–35 times. Not more than 7·5 sec. should elapse between the addition of acid and the extraction (see Fig. 5). With solution B the colour produced by oxaloacetate is destroyed by adding 6 ml. of 5N-HCl and placing the tube for exactly 8 min. in an ice-bath. The remaining colour is then extracted with 6 ml. of ethyl acetate. A reagent blank containing 2 ml. of 3% trichloroacetic acid instead of the tissue extract is subjected to each of the above procedures. For solution C both oxaloacetate and acetoacetate are destroyed by heating 2 ml. of the extract at 100° for 5 min. The solution is then cooled rapidly and treated in the same way as solution B and read against the appropriate reagent blank.

The optical density of the ethyl acetate layer is read at 450 mμ in a 4 cm. cell (model SP. 500 Unicam spectrophotometer). The bottom of the cell is raised, and the cell carrier appropriately masked, so that readings can be made with a 5–6 ml. sample. The difference in optical density between solution A and solution B is taken to represent the colour produced by reaction of the diazo reagent with oxaloacetate, and between solutions B and C that with acetoacetate.

Sensitivity of the method

Oxaloacetate and acetoacetate couple with diazotized *p*-nitroaniline to give compounds which have almost identical absorption spectra, as shown in Fig. 1. Under standard conditions all readings were made at 450 mμ. The time course of colour development with both compounds is given in Fig. 2. In order to minimize the colour contributed by acetoacetate, 20 min. was chosen as the reaction time in the standard method. The method can measure as little as 0·003 μmole of oxaloacetate (Fig. 3).

Specificity of the method

A major problem in utilizing this reaction is that of estimating oxaloacetate in the presence of acetoacetate. Decarboxylation of oxaloacetate by multivalent cations (Krebs, 1942) before reaction with the diazo reagent has proved satisfactory only with pure solutions of these compounds. In the presence of tissue extracts, the addition of multivalent cations decreases the colour produced with acetoacetate to a varying extent. A satisfactory separation can be obtained when the products of the coupling reaction are kept in acid at 0° for 8 min. As can be seen in Fig. 4, the coloured product obtained with acetoacetate is completely stable under these conditions, whereas that of oxaloacetate is nearly completely destroyed.

The addition of acid to the reaction mixture is essential to effect complete extraction of the resulting colour into the ethyl acetate layer. When

the extraction is performed within 7·5 sec. after the addition of acid (as with solution A) less than 5% of the colour obtained with oxaloacetate is lost; delaying the extraction for 20 sec. may result in a loss of as much as 20% (Fig. 5). Once extracted into ethyl acetate the colour yielded by both oxaloacetate and acetoacetate is stable for at least 6 hr.

A survey was made of compounds which might interfere with the estimation of oxaloacetate in tissues. The following 59 compounds (5 μmoles of each except where stated otherwise) failed to produce any significant colour with diazotized *p*-nitroaniline under the standard conditions: glycine, alanine, β-alanine, leucine, serine, threonine, cysteine, methionine, aspartic acid, asparagine, glutamic acid, lysine, arginine, histidine, phenylalanine, proline, hydroxyproline, glutathione, citrulline, glucose (50 μmoles), sucrose (50 μmoles), glucose 1-phosphate, fructose, sodium gluconate, carbamyl phosphate, 3-phosphoglyceric acid, α-glycerophosphate, glyoxal, oxalic, lactic, pyruvic, β-hydroxybutyric, citric, succinic, fumaric, malic, and laevulinic acid, sodium oleate, sodium crotonate, diphosphopyridine nucleotide, adenosine triphosphate, guanine, xanthine, thymine, uracil, acetoin, acetone, ethanol, urea, glycocyamine, calcium pantothenate, inositol, folic acid, vitamin B_{12}, calciferol, α-tocopherol, choline, glycerol and cholesterol. A number of substances (Table 1) react with diazotized *p*-nitroaniline, but under the standard conditions are not decolorized in acid, and so do not interfere with the estimation of oxaloacetate.

Several compounds tested give with diazotized *p*-nitroaniline a colour which is partially labile in acid under standard conditions (Table 2). Of these, only the members of the vitamin B_6 group are

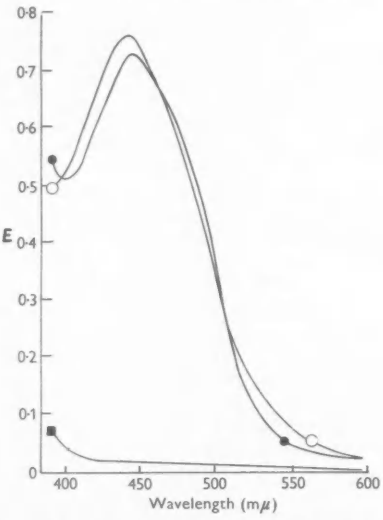

Fig. 1. Absorption spectra of the derivatives formed by coupling diazotized *p*-nitroaniline with oxaloacetate (○), and with acetoacetate (●). The products were formed by coupling diazotized *p*-nitroaniline with known samples of oxaloacetate and acetoacetate in 2 ml. of 3% trichloroacetic acid under standard conditions described for solution A in the text. The samples were read against ethyl acetate with the appropriate reagent blank (■) in a Carey recording spectrophotometer.

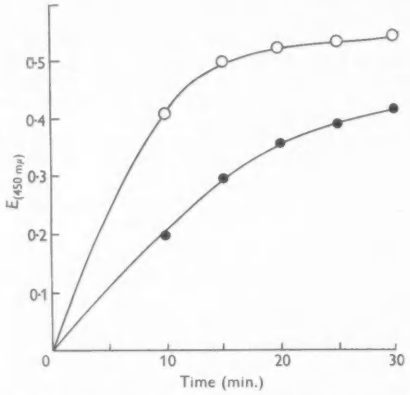

Fig. 2. Time curves for colour development with oxaloacetate (○) and with acetoacetate (●). Conditions were as described for solution A in the text.

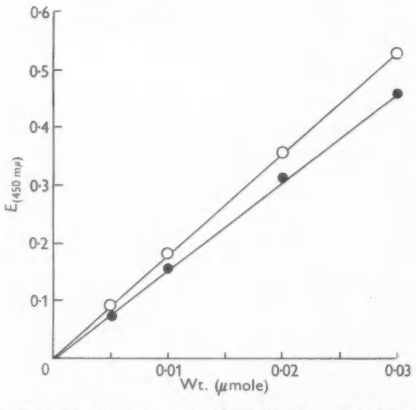

Fig. 3. Calibration curves for the estimation of oxaloacetic acid (○) and of acetoacetic acid (●). Conditions as described for solution A in the text.

present in sufficient quantity in rat liver (Rabinowitz & Snell, 1948) to provide slight interference. Since the major portion of vitamin B_6 occurring in tissues is tightly bound to protein (Clark, 1953; Siegal, Melnick & Oser, 1943; Rabinowitz & Snell, 1947) its interference is negligible (0·003–0·005 optical-density units).

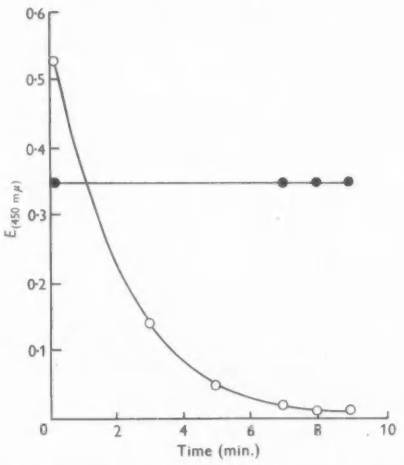

Fig. 4. Time curves for the stability in acid of the colours formed with oxaloacetate (○) and acetoacetate (●). Conditions were as for solution B in the text except for varying time in acid.

Table 1. *Substances which react with diazotized* p-*nitroaniline to give a coloured product which is stable in acid under the standard conditions defined in the text for solution B*

Substance	Amount (μmoles)	$E_{(450\,m\mu)}$
Ribonucleic acid*	40†	0·025
Deoxyribonucleic acid*	40†	0·010
Glycogen*	500†	0·025
Vitamin A acetate*	5	0·150
Ascorbic acid	5	0·032
Riboflavin*	1	0·025
Thiamine*	5	0·045
Tyrosine	1·1	0·049
Tryptophan	1·8	1·155
Ethanolamine*	5	0·023
Creatinine*	5	0·045
Diacetyl*	5	0·083
Acetoacetate	0·025	0·325
p-Hydroxyphenylpyruvate*	0·5	0·415
α-Oxoglutarate	1·0	0·037
Dihydroxymaleic acid	0·91	0·027

* Conditions were as for solution B as defined in the text except that the tubes were in acid at room temp. for 10 min. instead of at 0° for 8 min.
† Mg.

Table 2. *Substances which react with diazotized* p-*nitroaniline to give a coloured product which is labile in acid under the standard conditions defined in the text for solution B*

Substance	Amount (μmole)	$E_{(450\,m\mu)}$ Without acid	$E_{(450\,m\mu)}$ With acid
Oxaloacetate	0·03	0·530	0·000
α-Oxobutyrate	1·0	0·031	0·022
Malonic acid	1·0	1·315	1·275
Pyridoxine	0·1	0·410	0·317
Pyridoxamine	0·1	0·432	0·358
Pyridoxal	0·1	0·527	0·444

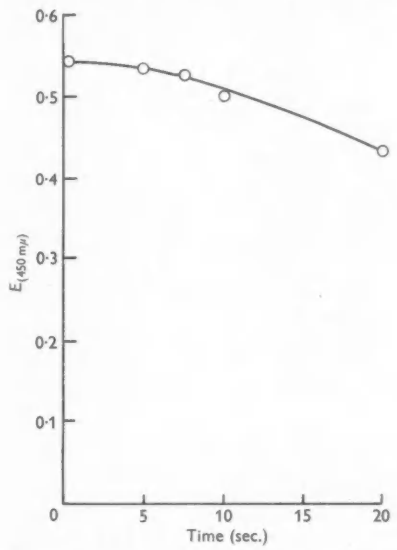

Fig. 5. Decrease in the colour of the oxaloacetate derivative during the first 20 sec. after the addition of acid. Conditions were as for solution A in the text except for the varying time at 0° before extraction.

Estimation of oxaloacetate in tissue preparations

As noted by Walker (1954), ascorbic acid interferes with colour development when acetoacetate is coupled with the diazo reagent. In fresh trichloroacetate extracts of normal liver there is sufficient ascorbate to interfere with the estimations of both acetoacetate and oxaloacetate (recoveries of added oxaloacetate and acetoacetate range from 60 to 80%). Walker (1954) removed ascorbate by the addition of cupric acetate to the extracts, but this treatment also destroys oxaloacetate. The difficulty was resolved by finding that ascorbic acid is completely removed by the addition of $NaNO_2$ for 30 sec. at acid pH (3% trichloroacetic acid),

whereas oxaloacetate under identical conditions is stable. This is illustrated in Fig. 6. With ascorbic acid thus eliminated, excellent recoveries of both acetoacetate and oxaloacetate are obtained (see Table 3).

Levels of oxaloacetate and acetoacetate in rat livers

Figs. 7 and 8 demonstrate that under the standard conditions adopted the colour produced is proportional to the amount of tissue analysed with the livers of both normal and fasted rats. Any significant variation in the technique may result in a non-linear relationship for solution B. This is particularly true for tissues from fasted animals.

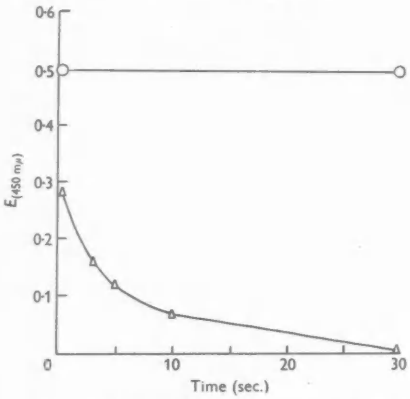

Fig. 6. Effect of adding $NaNO_2$ to a solution of oxaloacetate (0·03 μmole; ○) and of ascorbic acid (0·05 μmole; △) in 3% trichloroacetic acid for varying periods before the addition of the diazotized *p*-nitroaniline; conditions were otherwise as for solution A in the standard procedure.

Table 3. *Recovery of oxaloacetate and acetoacetate added to extracts of liver from normal and starved rats*

Extracts were prepared as described in the text and, after the additions indicated, were analysed by the standard method.

Addition to 2 ml. of extract (μmole)		Found (μmole)	
Oxaloacetate	Acetoacetate	Oxaloacetate	Acetoacetate
Normal rat liver			
—	—	0·003	0·004
0·02	—	0·022	—
—	0·047	0·004	0·051
0·02	0·047	0·021	0·051
Liver of rats starved 24 hr.			
—	—	0·004	0·034
0·02	—	0·024	—
—	0·017	0·002	0·051
0·02	0·017	0·022	0·053

Actual levels of oxaloacetate and of acetoacetate in the livers of normal and fasted rats are recorded in Table 4. It is of interest that the level of oxaloacetate in the livers of rats fasted 24 hr. is essentially the same as that in normal animals,

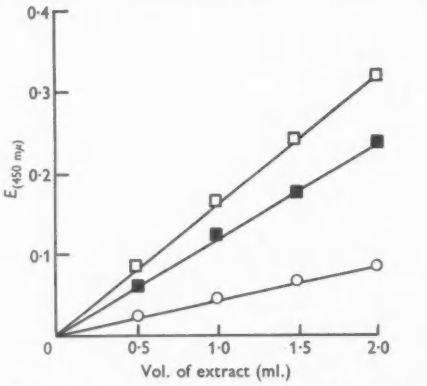

Fig. 7. Linearity of colour development with varying amounts of tissue from normal liver: □, solution A; ■, solution B; ○, oxaloacetate. Conditions were as for the standard method described in the text.

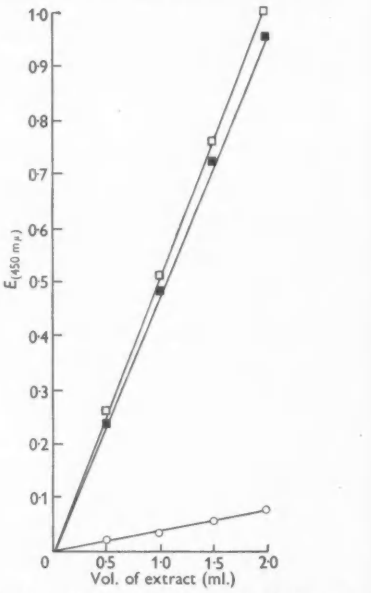

Fig. 8. Linearity of colour development with varying amounts of tissue from the livers of rats fasted for 27 hr.: □, solution A; ■, solution B; ○, oxaloacetate. Conditions were as for the standard method described in the text.

Table 4. *Estimation of oxaloacetate and acetoacetate in the livers of normal and fasted rats*

Estimations were made as described for the standard procedure in the text with 2 ml. of 20% trichloroacetic acid extract of normal livers and 1 ml. of 20% trichloroacetic acid extract of livers from fasted rats. Results are expressed as μmoles/100 g. dry wt. of tissue. Numbers in bold type are the means ±S.E.

Normal		Fasted, 24 hr.		Fasted, 69 hr.	
Oxaloacetate	Acetoacetate	Oxaloacetate	Acetoacetate	Oxaloacetate	Acetoacetate
6·3	3·6	6·0	91·4	<1	185·0
3·2	7·6	4·3	63·6	1·7	94·1
4·8	6·6	5·6	35·4	2·1	90·0
5·0	8·0	2·0	56·9	2·3	86·7
3·7	4·8	2·4	69·0	3·5	94·3
2·3	2·9	3·9	53·7	<1	112·3
3·0	7·8	5·3	70·8	<1	121·7
3·6	10·1	6·1	34·9	1·8	113·2
4·0	3·0	3·4	70·7	1·0	122·7
		4·8	62·1	3·9	84·0
		3·1	62·7	—	—
4·0±0·9	**6·0±2·2**	**4·3±1·2**	**61·0±11·5**	**1·8±0·9**	**110±21**

whereas in the same period acetoacetate has increased approximately tenfold. After fasting for 69 hr., there appears to be a decrease in the level of oxaloacetate. However, the colour due to acetoacetate in these livers is 20–50 times that contributed by oxaloacetate. Consequently, the results for oxaloacetate at 69 hr. may exceed the limit of reliability of the method.

DISCUSSION

The method described in this paper permits the accurate estimation of as little as 0·003 μmole of oxaloacetate. This exceeds the sensitivity of the usual manometric method by more than 100-fold (Krebs & Eggleston, 1945). The method appears to be sufficiently sensitive and specific for estimating the steady-state tissue levels of this compound. Although an extensive survey of biological compounds has failed thus far to reveal any that seriously interfere with the estimation of oxaloacetate, such may be found in further studies.

The oxaloacetate values for normal rat-liver tissue reported here are lower than those found by Frohman, Orten & Smith (1951*a*, *b*). However, the determination of oxaloacetate by these authors involved certain technical difficulties which could not be completely resolved.

The major weakness of the present method is that oxaloacetate is determined in the presence of acetoacetate. In most experimental circumstances, the concentration of oxaloacetate may be expected to be very low in relation to that of acetoacetate. The difficulty in estimating oxaloacetate when more than 0·06 μmole of acetoacetate is present in the sample will limit the general usefulness of the method. Attempts to separate the reaction products of oxaloacetate and acetoacetate by paper chromatography and a variety of solvent systems have been unsuccessful, owing to either inadequate separation or instability of the compounds.

The observation that the steady-state level of oxaloacetate is unchanged after fasting for 24 hr. is of interest. The two major paths for the disposition of acetyl-coenzyme A involve condensation either with acetyl-coenzyme A to form acetoacetate or with oxaloacetate to enter the tricarboxylic acid cycle. It has frequently been proposed that a diminished supply of oxaloacetate is one of the major factors contributing to the accumulation of acetoacetate in ketosis (cf. Lehninger, 1946; Krebs, 1948–9; Breusch, 1948; Weinhouse & Millington, 1951; Lynen, 1952–3; Chaikoff & Brown, 1954; Cantarow & Trumper, 1955; West & Todd, 1955). The present results indicate that significant ketosis can develop in the presence of normal levels of oxaloacetate in the liver. This appears to be in accord with the observation that the administration of succinate *in vivo* does not relieve ketosis in diabetic humans or rats (Lawrence, McCance & Archer, 1937; Deuel, Hallman & Murray, 1937; Terrell, 1938), and with the finding that the conversion of [^{14}C]acetate into $^{14}CO_2$ proceeds at nearly identical rates in the livers of normal, fasted and alloxan-diabetic rats (Masoro, Chaikoff, Chernick & Felts, 1950; Chernick & Chaikoff, 1951; Lyon, Masri & Chaikoff, 1952; Medes, Thomas & Weinhouse, 1952; Medes, Spirtes & Weinhouse, 1953). However, it should be emphasized that the present observations show only that the level of oxaloacetate in liver may remain within normal limits early in ketosis. Information concerning the rate of turnover or the total quantity of oxaloacetate available for condensation with acetyl-coenzyme A is not provided by this study.

SUMMARY

1. The colorimetric method for estimation of acetoacetate by reaction with diazotized *p*-nitroaniline has been adapted for measurements of oxaloacetate in amounts of 0·003–0·03 μmole.

2. The steady-state level of oxaloacetate in the livers of normal and fasted rats was found to be about 4 μmoles/100 g. dry wt. of tissue. The level was essentially unchanged in starvation when the level of acetoacetate increased tenfold.

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Oak-bark Tannins

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The barks of the two common oaks, the pedunculate oak (*Quercus pedunculata* Ehrh.) and the sessile oak (*Quercus sessiliflora* Salisb.) have been the traditional tanning material of Britain and northern Germany since medieval times. The principal botanical difference between the species is that the pedunculate oak has acorns on a long peduncle and stalkless or nearly stalkless leaves, whereas the sessile oak has sessile or stalkless acorns and petiolated leaves, but intermediate or hybrid forms are common. For commercial purposes, the timber and bark of these trees are not differentiated. Our experience of this tanning material shows that best-quality oak bark contains 12–16% of tannin [cf. Howes (1953) who gives 12–14%].

Little information on oak-bark phlobatannins is available, but (+)-catechin and (+)-gallocatechin have recently been isolated from this source (Mayer, 1956; cf. Casparis & Reber, 1929). The nature of the partial-type formula for oak-bark phlobatannins has now been investigated. Phlobatannins have recently been isolated in high yield from the extractives of *Acacia catechu* heartwood and harvested *Uncaria gambir* leaves (Hathway & Seakins, 1957*a*), which have the same analytical properties and similar elementary analyses and absorption spectra to those of the polymers of

catechin oxidation (Hathway & Seakins, 1955; 1957*b*; Hathway, 1958). There is therefore a strong supposition that these phlobatannins are formed by aerobic oxidation of catechin epimers by polyphenoloxidases in the heartwood and detached leaves respectively (Hathway & Seakins, 1957*a*). The isolation of the free plant phenolics and the purification of a phlobatannin fraction from oak bark, and an investigation of the polyphenoloxidase activity of the cambium, have been undertaken. The phenolics of mature leaves and the distribution of tannin extractives in stembark have also been explored.

EXPERIMENTAL

General. Evaporations were carried out in N_2 under reduced pressure at a temperature below 35°. Paper chromatography was carried out in all-glass apparatus in a constant-temperature enclosure at 25°. Chromatograms were dried at room temperature, unless otherwise stated. Melting points were determined on a Kofler block. Specific rotations were determined for the D line of sodium, with a 1 dm. microtube. Five pairs of readings with the solutions and five pairs with a solvent blank were taken in each case, at 20–25°.

Systematic separation of an 80% methanolic extract of oak bark into fractions of chemically similar substances

Slivers of stembark were stripped from a 20-year-old oak sapling immediately after felling. A portion was used for the analysis of moisture, and of tannin and non-tannin extractives (*Official Methods of Analysis*, 1957). Acids and salts were estimated by titration with 0·02N-NaOH on portions withdrawn from such an aqueous extract as that required by the *Official Methods*, before and after its passage through a Zeo-Karb 225 cation-exchange resin (2 mg.equiv./ml.) in the H^+ cycle. The stembark, which had a 60% moisture content, contained 12·6% of tannin and 6·9% of non-tannin extractives on the moisture-free bark. Each 100 g. of extractives contained 60 mg.equiv. of acid and 70 mg.equiv. of salt.

A portion (500 g.) of fresh bark was percolated with methanol (1·5 l.) for 5 days, after which the cell-wall fraction was removed by filtration through a sintered-glass funnel, and pressed and washed (5 × 100 ml.) with 80% methanol. Partial evaporation of the combined filtrate and washings left a soln. (400 ml.) which was continuously extracted for 4 days with peroxide-free ether in a Schacherl apparatus, ice–water being pumped through the condenser. Evaporation of the solvent left a solid residue which was dried over P_2O_5 at 20° *in vacuo* for 2 days. The ether-soluble fraction (4·3 g.) constituted 2·2% by wt. of the moisture-free bark.

Residual ether was removed from the aqueous phase, which was continuously extracted for 200 hr. with four changes of ethyl acetate in a Schacherl apparatus. Evaporation of the solvent left solid residues (5 g.) which constituted 2·5% by wt. of the moisture-free bark.

Residual ethyl acetate was removed from the aqueous phase, which was then percolated (40 ml./hr.) through a column (bed-volume, 20 ml.) of Zeo-Karb 225 cation-exchange resin (1·95 mg.equiv./ml.) in the H^+ cycle, which was washed free from acid. The total effluent (800 ml.) was evaporated to 500 ml., when a trace of insoluble material was removed by filtration through a few centimetres of Celite no. 535 (Johns-Manville Inc.) on a sintered-glass funnel. The filtrate contained 10% of tannin (hide-powder test).

Paper chromatography of the ether-, ethyl acetate- and water-soluble phenolics

A 2% methanolic solution of the fraction under investigation was spotted (5 μl.) at a distance of 2 cm. from both edges of the lower left-hand corners of Whatman no. 2 filter papers, 25·5 cm. square, and chromatographed by the ascending method with 3% (v/v) acetic acid containing 2% (v/v) formic acid as first-way solvent system. The solvent was irrigated to within 1 cm. of the upper edge of the paper, which was subsequently dried, and developed in the other dimension with butan-2-ol–acetic acid–water (14:1:5, by vol.) for 20 hr. With the water-soluble phenolics, 50% (v/v) acetic acid was used as the second-way solvent system. Phenolics were detected by dipping the papers in a 1:1 (v/v) mixture of 0·3% $FeCl_3$ and 0·3% $K_3Fe(CN)_6$ solutions, and by means of a spraying reagent consisting of a mixture of 1 vol. of 12N-HCl with 2 vol. of a 10% (w/v) solution of vanillin in ethanol.

Separation of the ether- and ethyl acetate-soluble phenolics by partition chromatography on silica gel

Chromatographic grade silica gel (100 g., mesh size 100–200; L. Light and Co. Ltd.) was stirred with two 1 l. portions of 2N-HCl at 100° for 2 hr.; the supernatant was then decanted, and the sediment washed free from acid to Congo red. The wet slurry was washed with 2 l. of methanol, air-dried and reactivated at 110° for 8 hr.

Suitable tubes were packed with the silica gel (80 g.) which had been triturated with water (52 ml.) and slurried with peroxide-free ether by Bradfield, Penney & Wright's (1947) method. The eluent was prepared by equilibration of peroxide-free ether with excess of water, followed by retention of the upper phase. The orange powder from the ether extraction (4·3 g.) was triturated with portions of wet ether (total, 100 ml.), and the solution decanted from insoluble material (500 mg.) was applied to the top of the column, which was subsequently percolated (2 ml./min.) with wet ether.

With the ethyl acetate-soluble phenolics, the column was packed with silica gel which had been triturated with the same proportion of water and slurried with ethyl acetate. The cream powder (3·5 g.), corresponding to the first two fractions in Table 2, was applied to the top of the column, which was eluted with wet ethyl acetate.

Isolation of leucodelphinidin

The first 100 ml. of eluate obtained from the column on which the ethyl acetate-soluble phenols were adsorbed contained an unresolved mixture of phenolics (2 g.). Evaporation of the next 200 ml. of eluate left 1 g. of *leucodelphinidin*, which separated in crystals, m.p. 155–160°, R_F 0·4 in butan-2-ol–acetic acid–water (14:1:5, by vol.) (Found: C, 53·0; H, 5·1. $C_{15}H_{14}O_8.H_2O$ requires C, 53·0; H, 4·7%) which afford delphinidin on treatment with acid. The *hepta-acetate* melted at 140–144° (Found: C, 56·5; H, 5·2. $C_{29}H_{35}O_{15}$ requires C, 56·0; H, 5·6%).

Investigation of the ethyl acetate-soluble fraction for leucoanthocyanins

A portion (20 mg.) of each ethyl acetate-soluble fraction and one broad-bean (*Vicia faba*) testa were treated with 3 ml. of 2N-HCl at 100° for 20 min., and the supernatant was decanted into a narrow test tube and extracted with enough *n*-amyl alcohol to give an upper phase of sufficient depth for spotting on starting lines, 3 cm. from the lower edges of Whatman filter paper no. 1, 57 cm. in length. The applications were repeated with intermittent drying, until the colour of each spot was intense enough to ensure visibility of the anthocyanidins on the developed chromatograms. Single-way ascending chromatography was effected with water–acetic acid–12N-HCl (10:30:3, by vol.), and *m*-cresol–5·5N-HCl–acetic acid (1:1:1, by vol.) solvent systems (Bate-Smith, 1954).

Purification of the phlobatannin fraction by cellulose-column chromatography

Solka Floc cellulose (Grade, BW 200) (Johnsen, Jörgensen, and Wettre Ltd., Wood Pulp Agents, 26, Farringdon St., London, E.C. 4) was stirred twice with large portions of 2N-HCl and washed free from acid to Congo red on a sintered-glass filter. The iron-free pulp was washed with methanol, air-dried and homogenized in acetone by a top-drive macerator, and the slurry was transferred to a chromatography tube made out of Pyrex pipe-line components. The column (60 cm. × 5 cm.) was developed with acetone, and stored for 48 hr. and developed under pressure with aq. 10% (v/v) formic acid until free from acetone. A volume (125 ml.) of the aqueous solution containing the water-soluble phenolics was applied to the top of the column, from which the sugars and mobile phenolics were eluted with 5 l. of 10% (v/v) formic acid. Oak-bark phlobatannin occupied a zone stretching from 2·5 to 7·5 cm. from the top of the column.

Fractionation of an 80% methanolic extract of oak leaves

Mature sun-leaves (80 g.), harvested in August, were plunged into ice-cold 80% methanol (500 ml.) and homogenized in a top-drive macerator for 5 min. The homogenate was filtered through sintered glass, and the filtrate was extracted with successive portions of light petroleum (b.p. 40–60°) until free from chlorophyll and related compounds. Partial evaporation of the lower phase afforded an aqueous solution which was successively extracted with ether and ethyl acetate. The ether-soluble fraction accounted for 0·25% (by wt.) of the leaves, and the ethyl acetate-soluble fraction for a further 0·5%. The extracts were examined for phenolics by conventional two-dimensional paper chromatography.

Isolation of (−)-epi*catechin from* Acacia catechu *heartwood.* Chips (100 g.) of *Acacia catechu* heartwood, extracted with acetone by the method of Rao & Seshadri (1948), afforded (−)-*epi*catechin (1 g.) which crystallized from water, forming needles, m.p. 243–244°, $[\alpha]_D$ −69° (*c*, 1; ethanol).

Epi*gallocatechin.* A specimen was made available for the purpose of chromatographic identification through the courtesy of Dr E. A. H. Roberts, of the Indian Tea Association, London, S.E. 1.

Acetone-dried powder of oak cambium. A 20-year-old sapling was felled, and the stembark was immediately peeled. Cambium detached from the freshly exposed surfaces of the bark and sapwood was plunged into 2 l. of acetone at below 0°, and sliced, and homogenized in a top-drive macerator. The homogenate obtained was filtered through sintered glass, and the solid (20 g.) was immediately washed five times with 500 ml. portions of ice-cold acetone and dried over paraffin wax at 0° *in vacuo*. The crude preparation had a polyphenoloxidase activity of purpurogallin number (P.N.) of 0·003. P.N. represents milligrams of purpurogallin formed from pyrogallol in 5 min. at 20°/mg. dry wt. of enzyme preparation. A Hilger Uvispek spectrophotometer was used for the P.N. determination by Keilin & Mann's (1938) method. This preparation was ground in a chilled Wiley mill, and homogenized five times with 500 ml. portions of ice-cold acetone, and filtered and dried over paraffin wax at 0° *in vacuo*. Wood fibres were removed. This powder (0·09 enzyme unit) had a P.N. of 0·005. One enzyme unit (E.U.) corresponds to the quantity of enzyme which produces 1 g. of purpurogallin in 5 min. at 20°.

Manometry. Use was made of Haldane's (1921) constant-pressure respirometer (Hathway & Seakins, 1957*a*). Both the reaction and the compensation vessels were shaken at 30±0·05° at a speed sufficient to maintain the reaction mixture as a foam.

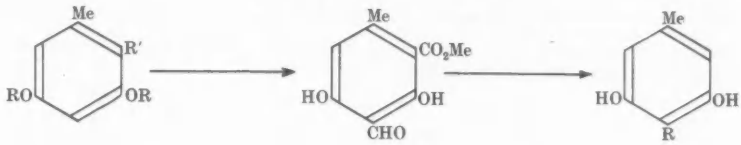
5-Methoxy-4-methylresorcinol. A specimen, m.p. 120° (Found: C, 62·1; H, 6·7. Calc. for $C_8H_{10}O_3$: C, 62·4; H, 6·5%), was prepared by the method of Robertson & Whalley (1951).

4-Nitrocatechol. A specimen, m.p. 174–175° (decomp.), was prepared from *o*-dibenzyloxybenzene by the method of Burton & Praill (1951).

Synthesis of 5-methylpyrogallol

Orcyl aldehyde (I), m.p. 180° (Adams & Levine, 1923), was converted into the dicarbomethoxy derivative (II), m.p. 84–85°, and oxidized by permanganate to dicarbomethoxyorsellinic acid (III), m.p. 132–134°, by the method of Hoesch (1913). Purdie methylation of III, followed by partial hydrolysis by the method of Robertson & Stephenson (1932), gave methyl orsellinate (IV), which crystallized from dilute methanol, forming prisms, m.p. 142°.

Preparation of methyl haematommate (V). Powdered, anhydrous $ZnCl_2$ was suspended in an ether soln. (300 ml.) of methyl orsellinate (7·1 g.); $Zn(CN)_2$ (11 g.) was added and HCl gas was conducted through the reaction mixture at 0° for 4 hr. The mother liquor was decanted from the crystalline aldimine hydrochlorides, which were washed four times with 100 ml. portions of absolute ether. The aldimine hydrochlorides were dissolved in 100 ml. of water and the soln. was heated at 100° for 40 min. After cooling for 16 hr., the product was collected and steam-distilled. Almost pure methyl haematommate (V) (3.1 g., 38% yield) separated from 5 l. of distillate at 0°, and crystallized from 100 ml. of methanol, forming glistening needles, m.p. 147° (Found: C, 57·1; H, 4·8. Calc. for $C_{10}H_{10}O_5$: C, 57·1; H, 4·8%), which were dried over P_2O_5 at 65° *in vacuo*. The non-volatile solid which separated from the residual liquor after steam-distillation, crystallized from water (charcoal), forming parallelepipeds, m.p. 130° (Found: C, 57·0; H, 4·6), of methyl *iso*haematommate, in 4% yield. Repetition of this preparation, with BF_3–ether complex



(V)

(I, R=H; R'=CHO)
(II, R=CO_2Me; R'=CHO)
(III, R=CO_2Me; R'=CO_2H)
(IV, R=H; R'=CO_2Me)

(VI, R=CHO)
(VII, R=OH)

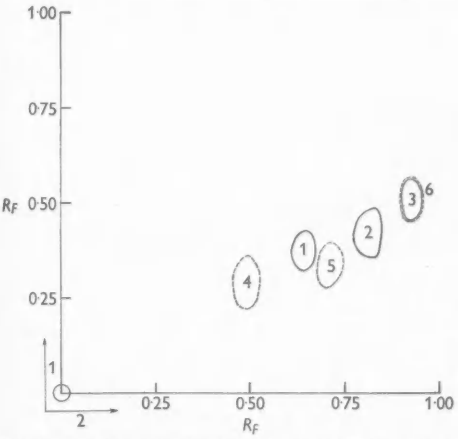


Fig. 1. Two-dimensional chromatogram of the ether-soluble phenolics. The material was applied in the position marked ⊙ and run first in 6% (v/v) acetic acid containing 2% (v/v) of formic acid, followed by butan-2-ol–acetic acid–water (14:1:5, by vol.). Spots located by $FeCl_3$–$K_3Fe(CN)_6$: 1, (+)-gallocatechin; 2, (+)-catechin; 3, unknown; 4, *epi*gallocatechin; 5, *epi*catechin; 6, methyl gallate.

(1 ml.) (Eastman Kodak Co., Rochester, U.S.A.) gave a 45% yield of methyl haematommate. Both preparations are improved variations of Curd, Robertson & Stephenson's (1933) preparation.

Hydrolysis of methyl haematommate gave atranol (VI), which crystallized from benzene in the form of pale-yellow needles, m.p. 124°, which were converted by the Dakin reaction into 5-methylpyrogallol (VII) by the method of Pfau (1926). 5-Methylpyrogallol crystallized from benzene, and sublimed at 105°/0·01 mm. Hg, forming long needles, m.p. 119° (Found: C, 60·1; H, 5·5. Calc. for $C_7H_8O_3$: C, 60·0; H, 5·8%), which were dried over P_2O_5 at 65° *in vacuo*.

In the aerobic oxidation experiments, 0·1 m-mole of 5-methylpyrogallol, and mixtures of 0·1 m-mole of this substance and 0·1 m-mole of phloroglucinol or 5-methoxy-4-methylresorcinol, underwent auto-oxidation at 30±0·05°, in 20 ml. of phosphate buffer, pH 7, for 2 days. Similar enzymic experiments were carried out in 3 hr.

RESULTS

The present work consists of a study *in vitro* of the aerobic oxidation of the (+)-gallocatechin and leucodelphinidin metabolites by oak-cambium polyphenoloxidase to a polymer comparable with the phlobatannin, which has now been isolated in 2% yield from the bark.

Young oak bark contains 50–60% of moisture and extractable 'tannins' account for about 12% of the dry bark. The bark was extracted with 80% methanol, the solvent removed and the phenolics and tannins in the resulting aqueous solution were fractionated by solvent extraction.

A two-dimensional chromatogram of the ether-soluble phenolics showed the presence of three constituents (Fig. 1). Two of these metabolites were chromatographically indistinguishable from catechin and gallocatechin respectively, and both compounds gave the vanillin reaction. The absence of *epi*catechin and *epi*gallocatechin was shown by the use of the authentic substances as markers. The third component did not react with vanillin, and was indistinguishable from methyl gallate on the chromatogram.

The ether-soluble phenolics were separated by partition chromatography on silica gel, with wet ether as eluent (Table 1). (+)-Catechin and (+)-gallocatechin were isolated from well-separated bands, and the physical properties and elementary analyses of these substances were in excellent agreement with previous findings (Freudenberg & Purrmann, 1924; Mayer, 1956). No crystalline material was obtained from the first band, however, which showed that the most mobile constituent was not methyl gallate.

The ethyl acetate-soluble phenolics were further fractionated on a time basis (Table 2). Two-dimensional chromatograms (Fig. 2) showed that the first two fractions contained three vanillin-positive constituents which were of lower R_F values in the second-way solvent system than those of the ether-soluble phenolics. All four fractions on treatment with warm acid generated mixtures of anthocyanidins, which were identified by single-

Table 1. *Separation of the ether-soluble phenolics by partition chromatography on silica gel*

Total recovery was 3·75 g. from an ether-soluble fraction weighing 3·8 g. A narrow pink band 1·0–1·5 cm. from the top of the column, and a yellow band somewhat lower were not investigated

Description of band	Eluate (ml.)	Phenolics recovered from eluates
1. Greenish brown	60	Oily residue (750 mg.) contained the phenolic, R_F 0·95 in butan-2-ol–acetic acid–water, but no crystalline material was obtained
2. Pink	500	(+)-Catechin (1·7 g.) crystallized from hot water (8 ml.) forming needles, m.p. and mixed m.p. 173–175°, $[\alpha]_D$ +8° (*c*, 1; acetone) (Found: C, 50·0; H, 5·8. Calc. for $C_{15}H_{14}O_6$, $4H_2O$: C, 49·8; H, 6·1%)
Absence of bands	200	—
3. Yellow	500	(+)-Gallocatechin (1·3 g.) crystallized from hot water (8 ml.), forming needles, m.p. 189–191°, $[\alpha]_D$ +15° [*c*, 1; 50% (v/v) acetone] (Found: C, 52·7; H, 5·6. Calc. for $C_{15}H_{14}O_7$,$2H_2O$: C, 52·7; H, 5·3%)

Table 2. *Ethyl acetate extraction of the aqueous extract*

Fraction	Extraction (hr.)	Residue (g.)	Examination of the residue by paper chromatography
1	75	2·8	Phenolic, R_F 0·4 in butan-2-ol–acetic acid–water, is dominant
2	25	0·7	
3	25	0·5	Phenolic, R_F 0·28 in the same solvent system, is dominant, and the tannins begin to appear on the chromatograms
4	75	1·0	
	Total 200		

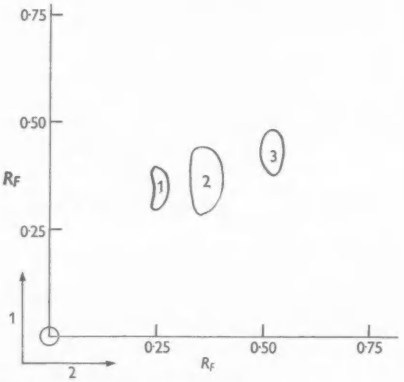


Fig. 2. Two-dimensional chromatogram of the ethyl acetate-soluble phenolics, the same solvent systems being used as in Fig. 1. Spots located by vanillin: 1, unknown; 2, leucodelphinidin; 3, unknown.

way paper chromatography in two different solvent systems as cyanidin and delphinidin (Bate-Smith, 1954), and the location of the absorption bands of these pigments on paper agreed with the recorded wavelengths. At least two of the constituents (Fig. 2) are therefore leucoanthocyanins, which also have the same hydroxylation pattern as the related cyanidin and delphinidin. The chromatogram in Fig. 2 shows that one constituent predominates in the first two fractions. This major constituent should therefore be capable of purification by partition chromatography. Despite the fact that the first band contained all three phenolics, crystalline leucodelphinidin was isolated from the second band, which was eluted from a silica gel column with wet ethyl acetate. This compound, which was characterized by a hepta-acetate derivative, gave delphinidin on treatment with acid. (+)-Catechin accounts for 1% of the dry weight of young oak bark, (+)-gallocatechin for up to 1% and leucodelphinidin for approximately 0·5%.

A two-dimensional chromatogram of the cation-free water-soluble extractives run first in N-acetic acid containing 2% (v/v) of formic acid, followed by 50% (v/v) acetic acid, revealed the presence of mobile, vanillin-positive phenolics, in addition to the phlobatannin which remained at the origin. The extract also contained free sugars. A portion of the extract was therefore adsorbed on the top of a large cellulose column, which was then eluted with 10% formic acid to remove contaminating sugars and mobile phenolics. The chemical nature of mixtures of phenolics present in fractions of effluent is described in Table 3. Since the anthocyanidins generated from these fractions by treatment with acid were separated as round spots on a paper chromatogram, it is unlikely that the corresponding leucoanthocyanins are polymeric. These leucoanthocyanins are probably glycosides.

Table 3. *Purification of the phlobatannin fraction by cellulose-column chromatography*

Vol. of eluate (l.)	Examination of mixed products
0·6–1·4	Sugar extractives (anthrone reagent)
1·0–1·4	Vanillin-positive phenolic, R_F 0·72 in 6% (v/v) acetic acid containing 2% (v/v) of formic acid, affords cyanidin by acid treatment
1·5–1·7	Vanillin-positive phenolic, R_F 0·64 in the same solvent system, affords cyanidin and delphinidin by acid treatment
1·8–2·9	Vanillin-positive phenolic, R_F 0·52 in the same solvent system, gives mostly delphinidin on acid treatment
3–5	Vanillin-positive phenolic, R_F 0·3 in this solvent system, gives mostly delphinidin on acid treatment
	Phlobatannin (1 g.) of low R_F was eluted with aq. 70% (v/v) formamide, pH 7, from a zone stretching from 2·5 to 7·5 cm. from the top of the column. The eluate, $\lambda_{max.}$ 285 mμ, was evaporated *in vacuo*, and the residue (Found: C, 53·3; H, 5·1. $C_{15}H_{10}O_7$, $2H_2O$ requires C, 53·3; H, 4·2%), which was washed with water and dried at 65° *in vacuo*, gave a vanillin reaction

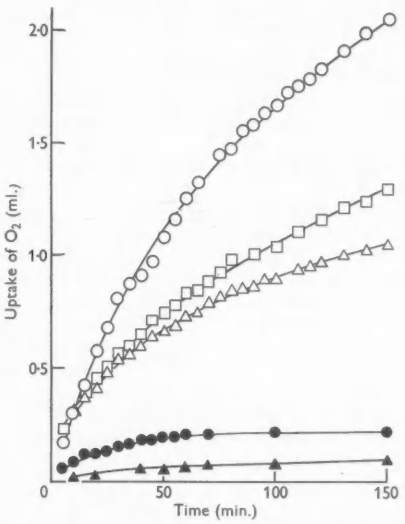


Fig. 3. Progress curves for the oxidation of gallocatechin, catechin and leucodelphinidin by oak-cambium polyphenoloxidase. In addition to 20 ml. of 0·07M-phosphate buffer, pH 7, and 5×10^{-4} E.U. of oak-cambium polyphenoloxidase (●), the reaction flask contained 0·1 m-mole of gallocatechin (○); catechin (△); leucodelphinidin (□); or 0·1 m-mole of catechin and 1 m-mole of 4-nitrocatechol (▲). A compensatory flask contained phosphate buffer.

The top zone of the column was eluted with 70% formamide (Hathway, 1956*a*). This eluate showed intense absorption at 285 mμ (Fig. 4) and, on evaporation, the phlobatannin, which accounted for 2% of the dry weight of young bark, analysed for $C_{15}H_{10}O_7,2H_2O$.

A methanolic extract derived from mature sun leaves was fractionated in the same way as the bark. Catechin and gallocatechin were identified in the ether-soluble fraction, and the three vanillin-positive phenolics in the ethyl acetate-soluble fraction. Besides these compounds some plant pigments, including quercetin, were present.

An acetone-dried powder of oak cambium which was substantially free from phenolics, had marked polyphenoloxidase activity. The crude enzyme preparation was tested with (+)-gallocatechin in phosphate buffer at 30° and pH 7, and the aerobic oxidation of this substance went smoothly to completion. At the level of enzyme concentration used, the rate of uptake of oxygen diminished very slowly during the course of reaction with this substrate. Such progress curves contrast with those for the aerobic oxidation of (+)-catechin by mushroom, potato and tobacco polyphenoloxidases (Hathway & Seakins, 1957*a*), as they show less enzyme inactivation. The enzyme concentrations in the present work were smaller than the previous ones, and these results suggest that the cambium enzyme is particularly suited to the oxidation of (+)-gallocatechin. Progress curves for the aerobic oxidation of (+)-catechin, (+)-gallocatechin and leucodelphinidin at the same enzyme concentration are recorded in Fig. 3. The rate of uptake of oxygen diminished much more rapidly with (+)-catechin

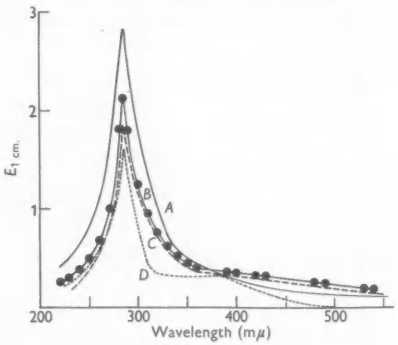


Fig. 4. Absorption spectra of oak-bark phlobatannin (*A*), and the aerobic oxidation polymers of (+)-gallocatechin (*B*), leucodelphinidin (*C*) and 5-methylpyrogallol with or without the addition of phloroglucinol or 5-methoxy-4-methylresorcinol (*D*). The spectra were measured in aqueous solution (pH 7).

and leucodelphinidin than with (+)-gallocatechin. Thus the (+)-gallocatechin metabolite is oxidized more rapidly than the other metabolites, and units derived from the oxidative polymerization of (+)-gallocatechin would therefore be expected to contribute extensively to the structure of oak-bark phlobatannin. The aerobic oxidation of catechin is arrested by the presence of Nakabayashi's (1954) 4-nitrocatechol inhibitor, at the relative concentrations of inhibitor to substrate which he employed. This inhibitor is efficient for catechol and catechin substrates.

The absorption spectrum of oak-bark phlobatannin exhibits a phenolic absorption band at 285 mμ and diminishing general absorption in the region 300–600 mμ. The absorption spectra of (+)-gallocatechin and leucodelphinidin-oxidation polymers are also precisely similar to that of oak-bark phlobatannin (Fig. 4).

The aerobic oxidation of an equimolecular mixture of 5-methylpyrogallol and phloroglucinol, and of 5-methylpyrogallol and 5-methoxy-4-methylresorcinol, gave polymers which showed a strong absorption band at 285 mμ and a shoulder at 350 mμ, which were identical to those in the spectrum of 5-methylpyrogallol-oxidation polymer alone (Fig. 4).

The analyses on bark detached from different parts of the stem of freshly felled trees show that the concentration of tannin diminishes from ground level to the crown (Table 4).

DISCUSSION

Examination of the leaves (of *Quercus*) shows the presence of (+)-catechin, (+)-gallocatechin and several vanillin-positive spots, some of which yield delphinidin on treatment with hot acid and may therefore be concluded to be leucodelphinidins. Besides these compounds some plant pigments, including quercetin, were also present. The occurrence of the (+)-catechin, (+)-gallocatechin and leucodelphinidin metabolites in the mature leaves suggests that in the Fagaceae they originate there, and Hillis (1956*a*, *b*) has reached the same conclusion about the origin of the leucoanthocyanins of certain eucalypts (cf. Jaccard, 1938). On account of Davis's (1955) microbial studies and important work involving the use of ^{14}C-labelled cinnamic and shikimic acids and L-phenylalanine (Brown & Neish, 1955; Geissman & Swain, 1957; Underhill, Watkin & Neish, 1957; Watkin, Underhill & Neish, 1957), it is probable that the (+)-catechin, (+)-gallocatechin and leucodelphinidin metabolites of *Quercus* spp. are formed in the leaves by reaction sequences of the sort considered by Neish and his co-workers and Hathway (1956*b*).

The bark extracts contain the same two catechins (which in this case were isolated in a crystalline state by column chromatography) and several leucoanthocyanins, one of which has also been obtained in a crystalline state and shown to be leucodelphinidin. This is the first time that a crystalline leucoanthocyanin yielding a commonly occurring anthocyanidin has been isolated. In addition to these crystalline compounds, several other low molecular-weight phenols are present, together with a large amount of phlobatannin ('condensed' tannin).

Examination of the cambium showed the presence of a polyphenoloxidase, which acted more readily on the pyrogallol type [(+)-gallocatechin and leucodelphinidin] phenols than on the catechol type [(+)-catechin] to form polymers.

From earlier work it appears likely that phlobatannin is formed from the oxidation of 'flavans' by polymerization through quinone. An examination of the possible linkage has therefore been made, since for catechin this is head-to-tail (Hathway, 1958; Hathway & Seakins, 1955; 1957*b*). With (+)-gallocatechin and leucodelphinidin as substrates for the cambium oxidase, a polymer is obtained which has a similar ultraviolet spectrum to that of the phlobatannin isolated from the bark, and is in contrast with the results obtained earlier with (+)-catechin and polyphenoloxidases (Hathway & Seakins, 1957*a*). The spectrum obtained is in fact similar to the ultraviolet spectrum of catechol-oxidation products (Hathway & Seakins, 1957*b*), and suggests strongly that tail-to-tail polymerization occurs. Confirmation of this hypothesis is obtained by the oxidation of 5-methylpyrogallol

Table 4. *Distribution of tannin in stembark*

Forest	Date of felling	Age (years)	Location on stem	Tannin percentage on moisture-free bark basis
Goytre	November 1955	20	Crown	11·5
			Butt	13·5
Chepstow	November 1955	20	Crown	11·3
			Butt	13·1
Dean	October 1955	70	Crown	7·8
			Centre	10·6
			Butt	11·0

with and without added phloroglucinol components. Aerobic oxidation of a mixture of 5-methylpyrogallol and 5-methoxy-4-methylresorcinol, and of 5-methylpyrogallol and phloroglucinol, by polyphenoloxidase, led to polymers which showed the characteristic absorption of the polymers of gallocatechin and catechol oxidation. 3-Hydroxy-5-methyl-*o*-benzoquinone and 5-methoxy-4-methylresorcinol or phloroglucinol do not therefore undergo oxidative coupling. Aerobic oxidation of (+)-gallocatechin by oak-cambium polyphenoloxidase proceeds by quinone-polymerization, and affords a product, the tail-to-tail polymer units of which account for the agreement in spectrum of the polymer with those of the catechol polymers including 5-methylpyrogallol polymer (cf. Hathway & Seakins, 1957*a*). A partial-type formula is shown in Fig. 5. The phlobatannin and the polymer obtained by the enzymic oxidation of (+)-gallocatechin have the analytical properties of a tannin (Gnamm, 1949; Schmidt, 1955). These polymers are precipitated from solution by gelatin or on refluxing with formalin–hydrochloric acid mixture, and are retained by hide powder (Grassmann, Endisch & Kuntara, 1951). The reaction of the polymer (and of oak-bark phlobatannin) with vanillin (Procter & Paessler, 1901) shows that polymerization does not involve the phloroglucinol residue of the monomer.

It is concluded therefore that the pyrogallol phenols are formed in the leaves, translocated to the cambium and undergo oxidation there, and the resulting phlobatannin (which is a tail-to-tail polymer) is stored in the bark. Confirmation of this is obtained in the distribution of 'tannins' in the stembark. The results on the distribution of 'tannins' in oak stembark agree with similar ones relating to the stembark of *Betula alba* (Jakimoff & Tolski, 1930), *Castanea mollissima* (Clarke, Steiner & Frey, 1942) and *Quercus laevis* (Rogers, Calderwood & Beebe, 1950) (Dicotyledonae), and *Larix siberica* (Egorov & Gershenzon, 1939), *Pinus echinata* (Snow, 1949), *P. ponderosa* (Kurth & Hubbard, 1951) and *P. radiata* (Anderson, 1952) (Gymnospermae). Field experiments involving girdled trees are in progress to test this supposition. The presence of phlobatannin in the bark and its absence from the heartwood implies that the cambium does not participate in the formation of heartwood extractives, and there is indirect evidence for this. Anderson (1953) found that the heartwood produced in pines, by stimulation of the cambium, did not contain all the phenols of normal heartwood, and Erdtman (1949) found a lignan in wound heartwood, which was different from that of normal spruce heartwood. The presence of gallocatechin and *epi*gallocatechin in the cacao bean (Forsyth, 1952) and tea leaf (Roberts, 1952) indicates that quinone polymerization accounts for phlobatannins which arise during subsequent fermentation. Low polymers derived from *epi*gallocatechin and *epi*gallocatechin gallate have recently been studied in connexion with tea fermentation (Roberts, 1957), and in agreement with the conclusions drawn from the present work, the evidence favours a tail-to-tail constitution. The isolation of mollisacacidin, 7:3′:4′-trihydroxyflavan-3:4-diol, from the heartwood (Keppler, 1957), and the detection of this and other leucoanthocyanins in the stembark of *Acacia mollissima* (Roux, 1957), are of probable significance in relation to the stembark tannins (Hathway & Seakins, 1957*a*). Related 3:4-dihydroxystilbene glycosides have been isolated by countercurrent extraction and column chromatography on nylon from amongst the cambium extractives of spruce (*Picea excelsa*) (Grassmann, Deffner, Schuster & Pauckner, 1956; Grassmann, Endres, Brockhaus & Merkle, 1957*a*; Grassman, Endres, Pauckner & Mathes, 1957*b*). Since a polyphenoloxidase has also been found in this tissue (W. Grassmann, personal communication), it is possible that the stembark tannins may be accounted for by the aerobic oxidation through quinone of such dihydroxystilbene aglycones by polyphenoloxidase. The phenolic and phlobatannin extractives of another important tannin material, *Eucalpytus redunca* heartwood, are now being investigated in this Laboratory. The present conclusion concerning the formation of oak-bark phlobatannins connects the formation of these compounds with the plant-browning reaction (Szent-Györgi & Vietorisz, 1931).

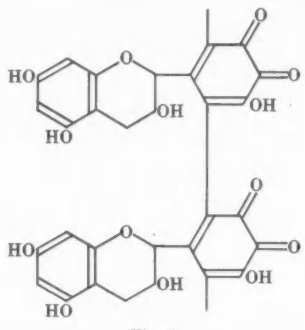


Fig. 5

SUMMARY

1. (+)-Catechin and (+)-gallocatechin, and leucodelphinidin, were respectively isolated from the ether- and ethyl acetate-soluble fractions of oak-bark phlobatannins. Leucodelphinidin,

$C_{15}H_{14}O_8,H_2O$, has been characterized by a hepta-acetate derivative. (+)-Catechin accounts for 1% of the dry weight of young bark, (+)-gallocatechin for up to 1% and leucodelphinidin for 0·5%.

2. Oak-bark phlobatannin, $C_{15}H_{10}O_7,2H_2O$, was isolated in considerable yield by cellulose-column chromatography, and exhibits an absorption band at 285 mμ and diminishing general absorption in the region 300–600 mμ.

3. Oak cambium exhibits polyphenoloxidase activity which is inhibited by 4-nitrocatechol. (+)-Gallocatechin was oxidized by this enzyme faster than (+)-catechin and leucodelphinidin.

4. (+)-Gallocatechin and leucodelphinidin oxidation polymers exhibit the same absorption spectrum, have the same analytical properties and similar elementary analyses to those of oak-bark phlobatannin.

5. Aerobic oxidation of mixed synthetic substrates, such as 5-methylpyrogallol and 5-methoxy-4-methylresorcinol, or 5-methylpyrogallol and phloroglucinol, by polyphenoloxidase gave polymers which showed the characteristic absorption of (+)-gallocatechin-oxidation polymer, oak-bark phlobatannin, and 5-methylpyrogallol-oxidation polymer.

6. The conclusion is drawn that phlobatannin is formed by the aerobic oxidation in the cambium of principally (+)-gallocatechin through a tail-to-tail quinone-polymerization mechanism.

7. The increase in tannin concentration of oak stembark from crown to butt suggests a downward movement of the phenolic metabolites from the leaves through the phloem.

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Studies on Phospholipids

3. DETERMINATION OF CHOLINE*

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Micromethods for the determination of choline have been described by Beattie (1936) and Glick (1944). Both procedures involved the use of Reinecke salt as a precipitant, required 20–150 μmoles of choline and depended on measurement of the pink colour of an acetone solution of the precipitate. More sensitive modifications of the reineckate method depend upon measurement of the chromium content of the precipitate (Marenzi & Cardini, 1943) or the absorption at 328 mμ (Winzler & Meserve, 1945). The use of potassium periodide as a choline precipitant was described first by Stanek (1905) and has since been modified by Erickson, Avrin, Teague & Williams (1940) and by Appleton, La Du, Levy, Steele & Brodie (1953).

Levine & Chargaff (1951) have described a semi-quantitative method involving chromatography on paper and subsequent treatment with phosphomolybdic acid. Choline shows up as a yellow spot which may be reduced with stannous chloride to give a blue colour.

The reaction between choline and phosphomolybdic acid has been investigated and this paper presents the results obtained and provides details of a method for the microdetermination of choline.

MATERIALS

Choline chloride. This material was recrystallized from *iso*butanol and dried over P_2O_5.

Choline phosphomolybdate. To 10 ml. of an aqueous solution containing 50 mg. of choline chloride was added 1 g. of phosphomolybdic acid dissolved in 5 ml. of water. The mixture was kept for 30 min. at 5° and the precipitated choline phosphomolybdate was removed by centrifuging. The precipitate was washed twice with *iso*butanol to remove excess of phosphomolybdic acid, and the solvent was removed by centrifuging and the choline phosphomolybdate dried *in vacuo*.

The solubility of choline phosphomolybdate in 0·1 N-HCl corresponded to 0·035 μmole of choline/0·5 ml. at 20° and 0·008 μmole of choline/0·5 ml. at 5°; in the presence of excess of phosphomolybdic acid the solubility would be still lower. Phosphomolybdic acid is freely soluble in *iso*butanol whereas the solubility of choline phosphomolybdate is less than 0·001 μmole/ml.; this solvent was therefore used for washing the precipitated choline salt. Choline phosphomolybdate contained 3 moles of choline/g.atom of P.

N-Dinitrophenylethanolamine and *N*-dinitrophenylserine were prepared as described by Wheeldon & Collins (1957).

Lecithin. This was prepared from rat-liver acetone-insoluble lipids by chromatography on alumina as described by Hanahan, Turner & Jayko (1951). The sample contained 110 μmoles of P, 3 μmoles of amino N and 105 μmoles of choline per 100 mg.

Kephalin. This was prepared from ox-brain lipids as described by Folch (1942) and corresponded with the fraction I of Folch. The sample contained 104 μmoles of P, 43 μmoles of amino N and less than 1 μmole of choline per 100 mg.

The rat-liver and brain lipids and the dinitrophenylated lipids were prepared as described by Wheeldon & Collins (1957).

Phosphomolybdic acid. A.R. grade was used. A saturated solution in water was clarified by centrifuging.

Stannous chloride solution. This consisted of a 5% (w/v) solution of $SnCl_2,H_2O$ in 10N-HCl.

METHODS

Phosphorus and amino N determinations were carried out as described by Wheeldon & Collins (1957).

Hydrolysis of samples. A sample (4–24 mg.) of fat (containing 2–12 μmoles of choline) was hydrolysed by heating at 100° with a mixture of 2 ml. of 6N-HCl and 2 ml. of ethanol. The mixture was placed in a flask with an air condenser so that for the first hour there was enough ethanol to maintain a homogeneous mixture, but over the course of the hydrolysis (18 hr.) the ethanol was gradually lost and the reaction was completed in an aqueous system. The hydrolysate was then evaporated to dryness *in vacuo* to remove the excess of HCl, 2 ml. of water was added and the pH adjusted to 6–7 by the addition of N-NaOH. The fatty acids were removed by extraction with diethyl ether; the aqueous solution was filtered, the filter washed with 5 ml. of water and the filtrate evaporated to dryness and the residue was dissolved in 2 ml. of water. Choline was determined on this solution.

Determination of choline as choline phosphomolybdate. A sample (0·5 ml.) of hydrolysate containing 0·3–3·0 μmoles of choline was mixed in a 15 ml. centrifuge tube with 0·1 ml. of a saturated aqueous solution of phosphomolybdic acid. The mixture was kept for 30 min. at 5° and then centrifuged; the supernatant was decanted and the tube was inverted and allowed to drain for 15 min. The precipitate

* Part 2: Collins & Wheeldon (1957).

was then suspended in 1 ml. of *iso*butanol and centrifuged, the supernatant was discarded and the process was repeated. The washed precipitate was then dissolved in 3 ml. of acetone. To this solution was added 1 ml. of 10 N-H_2SO_4 and 0·05 ml. of $SnCl_2$ solution and the mixture made up to 10 ml. with ethanol. The optical density of the blue solution was measured in a Hilger Spekker absorptiometer with an Ilford 608 filter. The term 'molecular-extinction coefficient' as used in this paper refers to the value obtained with the optical density thus measured and is approximately 87% of the true value at 630 mμ. The plot of concentration (0·3–3·0 μmoles/0·5 ml.) against the optical density was linear and passed through the origin. The value of the 'molecular-extinction coefficient' was 4810±170 (mean of 24 determinations).

RESULTS

Interfering substances. Serine and ethanolamine did not interfere provided that their concentrations did not exceed 20 μmoles/0·5 ml. This amount of serine or ethanolamine was equivalent to 16 mg. of phosphatidylserine or phosphatidylethanolamine and the sample for choline determination should not contain more than this amount of kephalin. Ammonia interfered with the method but was not present in lipid hydrolysates prepared as described by Wheeldon & Collins (1957). The presence of a reducing sugar, such as glucose, did not cause any reduction of phosphomolybdic acid and thus did not interfere. If the lipid was hydrolysed in an alkaline medium the presence of certain cations complicated the procedure. Na^+ ions produced no precipitate but K^+ (if more than 20 μequiv./0·5 ml.), Ba^{2+} and Ca^{2+} ions formed insoluble salts with phosphomolybdic acid, which, however, differed from choline phosphomolybdate in that they were insoluble in acetone.

Application to lipid hydrolysates. The recovery of choline added to lipid hydrolysates was quantitative, as is shown in Table 1. As a further check on the recovery of choline a sample of kephalin was mixed in varying proportions with a sample of lecithin, the total weight of the mixture being kept constant at 2·61 mg. The plot of the percentage of lecithin (100, 80, 50, 20 and 0) against the amount of choline found was linear, showing that the determination of choline was unaffected by the presence of kephalin. In the determination of choline in lipid hydrolysates the agreement between duplicates was 2·4% (fiducial limits for $P=0·95$).

Table 1. *Recovery of choline added to a lipid hydrolysate*

Known amounts of choline were added to a 0·5 ml. sample of a rat-liver lipid hydrolysate containing 1·06 μmoles of choline and the total choline was estimated by the phosphomolybdate method.

Sample	Choline added (μmoles)	Total choline found (μmoles)	Recovery (%)
1	0·28	1·34	100
2	0·83	1·88	99
3	1·11	2·13	97
4	1·39	2·44	99
5	1·67	2·68	97

Comparison with reineckate procedure. A comparison with the results obtained by precipitation as choline phosphomolybdate and as choline reineckate, by the procedure of Glick (1944), showed close agreement. The ratio of the choline content of three samples of rat-brain lipids and three of rat-liver lipids (each in duplicate) as determined by the reineckate to that determined by the phosphomolybdate method was 1·06±0·034. The 'molecular-extinction coefficients' used in the phosphomolybdate and reineckate methods were 4810±170 and 108±3 respectively. There is thus an uncertainty in this ratio of 4·5% due to the uncertainty in the extinction coefficients alone.

Application to dinitrophenylated lipids. Dinitrophenylethanolamine and dinitrophenylserine were not precipitated by phosphomolybdic acid at any concentration, but when they were heated with 6 N-HCl for 18 hr. there was some production of a reducing material which resulted in the formation of molybdenum blue on the addition of phosphomolybdic acid. The application of the unmodified method to dinitrophenylated lipids led to high results for choline, probably due to the adsorption of the molybdenum blue onto the precipitated choline phosphomolybdate. When the period of heating was 3 hr. there was no interference. Hydrolysis with N-HCl in ethanol was found to split off the dinitrophenylated bases quantitatively without any decomposition, and the following procedure for the determination of choline in dinitrophenylated lipids was developed.

The sample of dinitrophenylated lipid (5–25 mg.) was hydrolysed by heating with 5 ml. of N-HCl in ethanol under reflux for 3 hr. The mixture was evaporated to dryness *in vacuo* to remove excess of HCl, 1 ml. of water was added and the aqueous mixture was extracted three times with 5 ml. of light petroleum (b.p. 40–60°). The aqueous layer was then extracted with three 5 ml. portions of diethyl ether and the ethereal extracts were discarded. The light-petroleum extract after removal of solvent was combined with the aqueous solution, 1 ml. of 10 N-HCl was added to bring the normality to approximately 6 and the hydrolysis was continued by heating at 100° for 10 hr. From this point the procedure was the same as in the normal method.

There was no loss of P (see Table 2) involved when the ethereal solution containing the dinitrophenylated bases was discarded, although there was

Table 2. *Distribution of phosphorus between light petroleum, diethyl ether and water after hydrolysis of a dinitrophenylated lipid*

Four samples of a dinitrophenylated lipid from rat liver were hydrolysed and extracted as described in the text. P was determined in each of the three fractions.

	Distribution of P (%)			
Wt. of lipid (mg.)	3·4	4·6	4·2	5·8
Light-petroleum extract	3	13	16	15
Diethyl ether extract	<1	1	4	2
Aqueous solution	97	86	80	83

still unhydrolysed phospholipid in the light-petroleum extract. The application of the method to samples of fat before and after treatment with 1-fluoro-2:4-dinitrobenzene showed that the ratio of the choline content of dinitrophenylated fat to that of the native fat was $0{\cdot}99 \pm 0{\cdot}06$ (seven samples).

Choline contents of rat-liver lipids. The choline content of 18 samples of rat-liver lipids was $20{\cdot}4 \pm 3{\cdot}6$ (S.D.) μmoles/g. of fresh tissue. Artom (1945) found $23{\cdot}5 \pm 0{\cdot}8$ (S.D.) μmoles/g. of fresh tissue. Assuming a molecular weight of 800 these figures correspond to 1·6 and 1·9% of choline phospholipids in the moist liver respectively. Artom & Fishman (1943) and Cardini & Serantes (1942) respectively obtained values of 1·9 and 2·2% of choline phospholipids in moist liver.

DISCUSSION

The method for the determination of choline described in this paper has several advantages and is as sensitive as alternative methods. The reagent is light-stable, unlike ammonium reineckate solutions, and the precipitate is stable, in contrast with choline enneaiodide. With choline phosphomolybdate the final colour absorbs maximally at 630 mμ and is not subject to interference from impurities which interfere with measurements in the ultraviolet region. Levine & Chargaff (1951) showed that the only material from lipid hydrolysates which reacted with phosphomolybdic acid was choline and therefore the specificity of the method as applied to phospholipids is equal to that of other methods involving a precipitation reaction. For a given amount of choline the precipitate obtained with phosphomolybdic acid was the largest; for example 15 μg. of choline corresponds to 60 μg. of choline reineckate, 160 μg. of choline enneaiodide and 260 μg. of choline phosphomolybdate. The procedure described for the determination of choline has been applied to a large number of lipid samples and has given results that agree with those obtained by other methods.

SUMMARY

1. A colorimetric method for the determination of choline in lipids has been developed in which the choline in a hydrolysate is precipitated by phosphomolybdic acid.

2. Choline added to a lipid hydrolysate was quantitatively recovered, as was also the choline in mixtures of lecithin and kephalin.

3. The method agreed with the reineckate method but was not affected by light and is simpler to use.

We wish to express our appreciation of the technical assistance given by Mrs M. Labutis.

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Studies on Phospholipids

4. DETERMINATION OF ETHANOLAMINE AND SERINE*

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A reliable and sensitive method for the determination of ethanolamine and serine, the bases present in kephalin, is necessary in the study of phospholipid metabolism. The procedure described by Artom (1945) is reliable but not sensitive (range 10–50 μmoles), whereas that described by Levine & Chargaff (1951) is sensitive (range 0·1–0·3 μmole) but unreliable. The method devised by Axelrod, Reichenthal & Brodie (1953) involves the hydrolysis of the fat, the reaction of the free bases with 1-fluoro-2:4-dinitrobenzene (FDNB), the separation of the *N*-dinitrophenyl (DNP) bases and their quantitative estimation by their light absorption at a wavelength at which FDNB does not interfere. The present paper describes a method which involves the reaction of the lipids with FDNB, the complete removal of excess of reagents, the hydrolysis of the DNP-lipids and the determination of DNP-ethanolamine and DNP-serine by a differential colour reaction which does not involve their separation.

MATERIALS

All solvents used were reagent grade. The tetramethylammonium hydroxide was used as supplied as a 25% (w/v) solution in water. DNP-ethanolamine and DNP-serine were prepared as described by Wheeldon & Collins (1957).

N-(2:4-Dinitrophenyl)-ethanolamine-O-phosphoric acid. This was prepared by treating ethanolamine-*O*-phosphoric acid (423 mg.) (Californian Foundation for Biochemical Research) with FDNB (700 mg.) and $NaHCO_3$ (1 g. in 20 ml. of water). The mixture was left at 20° for 16 hr. in the dark and extracted with diethyl ether. The aqueous layer was cooled and acidified with 10N-HCl until an oil separated. This solidified at −10° and crystallized from ethanol. The yield was 0·24 g., m.p. 163–165° (uncorr.) (Found: C, 31·7; H, 3·4; N, 13·0; P, 9·6. $C_9H_{10}O_9N_3P$ requires C, 31·3; H, 3·3; N, 13·7; P, 10·1%). In 0·1N-HCl $\lambda_{max.}$ was at 350 mμ (ϵ = 15 500). On paper chromatography a single spot was obtained.

N-(2:4-Dinitrophenyl)-serine-O-phosphoric acid. This was prepared by treating serine-*O*-phosphoric acid monohydrate (406 mg.) (Californian Foundation for Biochemical Research) with FDNB (1 g.) and $NaHCO_3$ (1·5 g. in 30 ml. of water). The mixture was left for 16 hr. at 20° in the dark. The solution was then extracted with diethyl ether to remove excess of FDNB, acidified (pH 5) with 10N-HCl and concentrated to a syrup *in vacuo*. The free acid did not crystallize readily but on the addition of an equimolecular amount of triethylamine the salt was crystallized from propan-2-ol. The yield was 0·40 g., m.p. 147° (uncorr.) (Found: C, 39·1; H, 5·9; N, 12·0; P, 7·2. $C_9H_{10}O_{10}N_3P$, $C_6H_{15}N$ requires C, 39·9; H, 5·5; N, 12·4; P, 6·9%). In 0·1N-HCl $\lambda_{max.}$ was at 350 mμ (ϵ = 14 900). On paper chromatography a single spot was obtained.

* Part 3: Wheeldon & Collins (1958).

METHODS

The extraction of the lipids and their dinitrophenylation was carried out as described by Wheeldon & Collins (1957).

Paper chromatography. Ascending chromatography of the DNP-bases was carried out on Whatman no. 1 paper either with M-NaH_2PO_4 and 0·5M-Na_2HPO_4 (Levy, 1954) or with *tert.*-pentanol–pyridine–water (5:1:5, by vol.).

RESULTS

Hydrolysis of N-(2:4-dinitrophenyl)-lipids. The most satisfactory medium for hydrolysis of the DNP-lipids was ethanolic N-HCl; hydrolysis was complete in 3 hr. at 100°. Table 1 shows that the recoveries of total DNP-bases after hydrolysis of DNP-lipids were quantitative. The hydrolysis procedure was complicated by the fact that DNP-serine in ethanolic N-HCl formed the ethyl ester and that DNP-serine was light-sensitive. The

Table 1. *Recovery of N-(2:4-dinitrophenyl)-bases after the hydrolysis of N-(2:4-dinitrophenyl)-lipids*

DNP-lipids were hydrolysed by heating with N-HCl in ethanol for 3 hr. The amount of DNP-bases was determined from the light absorption; DNP-lipids were measured in light petroleum and the free DNP-bases were measured in water after removing the fatty acids.

	DNP-base (μmoles)		
Lipid	Before hydrolysis	After hydrolysis	Recovery (%)
Sheep brain	6·0	5·9	98
Sheep brain	32·6	32·3	99
Guinea-pig brain	60·5	61·3	101
Rat liver	3·7	3·4	92
Rat liver	8·3	8·7	105

solutions were shielded from light. As the ethyl ester of DNP-serine could not be distinguished from DNP-ethanolamine in the colour reaction to be described the initial hydrolysis in ethanolic N-HCl was followed by hydrolysis in aqueous N-HCl. The bases present, after hydrolysis, were identified by paper chromatography. In no case were there more than 2% of DNP-compounds other than DNP-ethanolamine and DNP-serine, and these impurities were in the positions expected for the corresponding phosphoric acid derivatives. The amounts present were estimated by cutting out the spots, and eluting with water and measuring the absorption at 350 mμ. Table 2 gives the R_F values for the DNP-bases and the phosphoric acid derivatives.

Colour reaction for N-(2:4-dinitrophenyl)-bases. Porter (1955) described the reaction between tetramethylammonium hydroxide dissolved in dimethylformamide and various nitroderivatives of aromatic compounds. DNP-ethanolamine and DNP-serine in this system gave rise to coloured ions with $\lambda_{max.}$ at 430 mμ having dissociation constants of 0·1 and 6 mM respectively and which on the addition of 20% (v/v) ethanol became 3 mM and 0·2 M respectively. Fig. 1 shows the effect of varying concentrations of tetramethylammonium hydroxide on the values of the molecular-extinction coefficient at 500 mμ. At a concentration of quaternary base of 27 mM the DNP-ethanolamine is completely ionized whereas the DNP-serine is only 10% ionized, and this difference forms the basis of the present method. Fig. 2 shows the absorption spectra of the two DNP-bases under these conditions and demonstrates that 500 mμ is a convenient wavelength to use. Measurements at 393 mμ, where the two compounds have the same molecular-extinction coefficient, enable the combined concentration of the DNP-bases to be calculated.

The reliability of the colorimetric procedure depends on the concentration of tetramethylammonium hydroxide being the same for unknown and for standards. In a hydrolysate of a DNP-lipid the

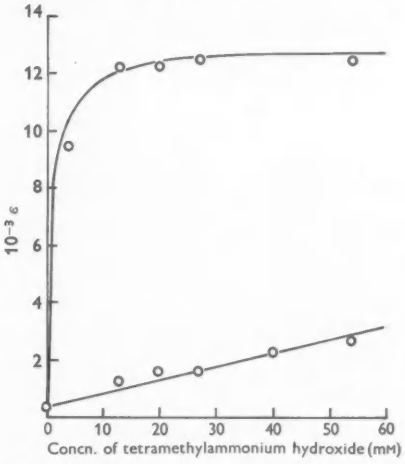

Fig. 1. Colour development and concentration of quaternary base. Molecular-extinction coefficients at 500 mμ of DNP-ethanolamine and DNP-serine were measured in dimethylformamide–ethanol (4:1, v/v) and known amounts of tetramethylammonium hydroxide. Theoretical curves have been calculated assuming a dissociation constant of 3 mM for DNP-ethanolamine (upper curve) and of 0·2 M for DNP-serine (lower curve); experimental values are indicated by the circles.

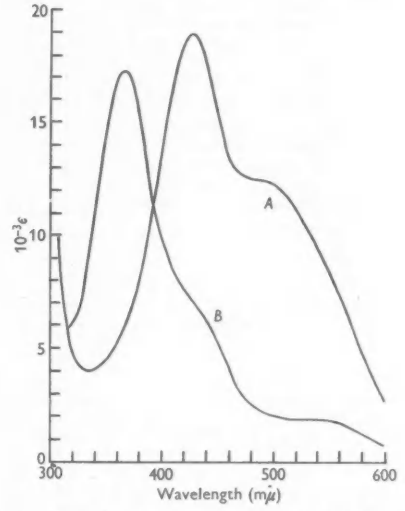

Fig. 2. Absorption spectra of DNP-ethanolamine and DNP-serine in 27 mM-tetramethylammonium hydroxide. DNP-ethanolamine (curve *A*) and DNP-serine (curve *B*) were dissolved in a mixture of dimethylformamide–ethanol–*n*-butylamine (4:1:0·04, by vol.).

Table 2. *R_F values for some N-(2:4-dinitrophenyl)-bases related to N-(2:4-dinitrophenyl)-lipids*

The solvent system used was *A*, that described by Levy (1954) or *B*, *tert.*-pentanol–pyridine–water (5:1:5, by vol.). Whatman no. 1 paper was used in an ascending system. Time of run, 18 hr.

	R_F in solvent system	
	A	*B*
DNP-ethanolamine	0·28	0·90
DNP-serine	0·46	0·38
DNP-ethanolamine-*O*-phosphoric acid	0·60	0·24
DNP-serine-*O*-phosphoric acid	0·80	0·10

water-soluble fraction will contain, in addition to the DNP-bases, phosphoric acid, glycerophosphoric acid, inositol phosphoric acid, choline phosphate or chloride and free hydrochloric acid. The last component is removed by evaporation *in vacuo*. If the concentration of tetramethylammonium hydroxide were to change by 10%, i.e. from 27 to 24 mM, it can be calculated from the equations given below and from the data in Fig. 1 that the error introduced into the method will not exceed 2%. This change in concentration could be caused by the presence of 30 μg.equivalent of acid (or 15 μg.atoms of P) in the hydrolysate, and the sample of hydrolysate taken for analysis should not contain more than 15 μg.atoms of P.

The addition of 0·8% (v/v) of *n*-butylamine to the solvent system was found to assist in buffering the solution.

Determination of ethanolamine and serine in lipids

On the basis of the foregoing results the following procedure is recommended. The lipids are obtained from the tissues and dinitrophenylated as described by Wheeldon & Collins (1957). A quantity of DNP-lipid which corresponds to between 0·1 and 1·5 μmoles of amino N is dissolved in 5 ml. of ethanolic N-HCl and hydrolysed by boiling under reflux for 3 hr. The hydrolysate is evaporated to dryness *in vacuo*, 2 ml. of N-HCl is added and the mixture is again refluxed for a further 3 hr. After adding about 0·1 g. of Hyflo Supercel the mixture is filtered, the filter is washed with 5 ml. of water and the filtrate is reduced to dryness by heating *in vacuo*. The residue is dissolved in 10 ml. of a mixture of dimethylformamide–ethanol–*n*-butylamine (4:1:0·04, by vol.), 0·1 ml. of 25% (w/v) tetramethylammonium hydroxide is added and, after mixing, the extinctions are measured at 393 and 500 mμ. If e and s respectively represent the amounts of DNP-ethanolamine and DNP-serine in μmoles in the 10·1 ml. of solution and if E_1 and E_2 represent the extinctions respectively at 393 mμ and at 500 mμ then it follows that $E_1 = ae/1{\cdot}01 + bs/1{\cdot}01$ and $E_2 = ce/1{\cdot}01 + ds/1{\cdot}01$. The constants a, b, c and d are equal to 10^{-4} times the molecular-extinction coefficients; the mean values and standard errors (12 determinations) are: a, 1·13±0·007; b, 1·13±0·007; c, 1·23±0·007 and d, 0·184±0·006. Hence

$$e = 0{\cdot}965E_2 - 0{\cdot}157E_1;$$

$$s = 1{\cdot}05E_1 - 0{\cdot}965E_2;$$

$$e + s = 0{\cdot}894E_1$$

and $$e/(e+s) = 1{\cdot}18 - 1{\cdot}08E_2/E_1.$$

The recoveries from mixtures of known amounts of DNP-ethanolamine and of DNP-serine are shown in Table 3. Known amounts of DNP-serine were added to hydrolysates of guinea-pig lipids and, as shown in Table 4, the average recovery was 104·5%.

Reaction of other N-(2:4-*dinitrophenyl*)-*compounds*. The phosphoric acid derivatives of DNP-ethanolamine and DNP-serine, and dinitrophenylated lipids, gave $\lambda_{max.}$ at 365 mμ in the colour test whereas the methyl ester of DNP-serine, and methylated and dinitrophenylated lipids (Collins & Wheeldon, 1957), gave $\lambda_{max.}$ at 430 mμ.

Occurrence of ethanolamine and serine in some lipids. The proportions of ethanolamine and serine were determined on a number of lipids, with the results shown in Table 5.

DISCUSSION

The method described in this paper for the determination of serine and ethanolamine has certain features in common with the procedure described by Axelrod *et al.* (1953). Although it is as convenient to treat the lipid with FDNB before hydrolysis as afterwards (cf. Axelrod *et al.* 1953), the former procedure has the advantage that removal of the excess of reagents is readily

Table 3. *Application of the colour reaction to mixtures of* N-(2:4-*dinitrophenyl*)-*ethanolamine and* N-(2:4-*dinitrophenyl*)-*serine*

Known amounts of DNP-ethanolamine and DNP-serine were mixed and analysed. All figures are in μmole.

DNP-serine		DNP-ethanolamine	
Added	Found	Added	Found
0·84	0·85	0·24	0·23
0·56	0·60	0·48	0·47
0·28	0·32	0·72	0·72

Table 4. *Recoveries of* N-(2:4-*dinitrophenyl*)-*serine added to hydrolysates of* N-(2:4-*dinitrophenyl*)-*lipids*

DNP-lipids prepared from guinea-pig tissues were hydrolysed and the DNP-bases determined before and after adding known amounts of DNP-serine. All figures in μmole.

		DNP-serine		
Lipid	DNP-ethanolamine	In sample	Added	Recovered
Brain	0·47	0·06	0·22	0·24
Kidney	0·58	0·06	0·22	0·22
Liver	0·85	0·10	0·11	0·13
Heart	0·37	0·06	0·11	0·10

Table 5. *Ethanolamine and serine contents of some lipids*

All figures are μmoles/100 mg. of lipid.

Source	Ethanolamine	Serine
Guinea-pig kidney	12	1·4
Guinea-pig liver	21	2·6
Guinea-pig heart	11	2·1
Guinea-pig brain	23	3·5
Rat brain	19	6·5
Sheep brain	24	8·9
Egg yolk	18	<0·2
A serine-rich fraction of ox-brain lipids	2·4	14

accomplished. Further, the use of a differential colour reaction is less time-consuming than alternative methods which involve separation of the two DNP-bases (cf. Axelrod *et al.* 1953).

In the rare instances where more than two DNP-bases are present, as, for example, in *Neurospora* lipids, which contain ethanolamine, serine and 1-amino-2-methylpropan-2-ol (Ellman & Mitchell, 1954), the procedure described by Levy (1954) for DNP-amino acids can be used. When this method was applied to a mixture of DNP-ethanolamine and DNP-serine the recovery was good (98%) but the colorimetric method was quicker.

The experimental results on the application of the colour test to the DNP-bases suggest that the acidic group in the DNP-serine is responsible for the difference in the dissociation constants of the coloured forms. This is confirmed by the fact that the ethyl ester of DNP-serine reacts like DNP-ethanolamine and that DNP-ethanolamine-*O*-phosphoric acid reacts like DNP-serine. These observations also explain why the colour reaction with DNP-lipids simulates DNP-serine and methylated DNP-lipids simulate DNP-ethanolamine, as in the first case the phosphoric acid groups are free and in the second case they are in the form of methyl esters.

The procedure described in this paper was developed as an adjunct to the study of DNP-lipids (Collins & Wheeldon, 1957) but, if used in conjunction with the method described by Wheeldon & Collins (1957) for total amino N, should be of wide applicability.

SUMMARY

1. A method depending on a differential colour reaction for the determination of ethanolamine and serine in phospholipids is described.

2. The advantages of the method, as compared with those of other methods, are discussed.

Our thanks are due to Mrs M. Labutis for her technical assistance.

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Substrates for Rumen β-Glucosidase

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Substrates used for β-glucosidase include natural products such as cellobiose and salicin as well as synthetic compounds like the nitrophenyl glucosides (Aizawa, 1939; Conchie, 1954). Enzyme extracts from single micro-organisms have been shown to vary in their specificities. Jermyn (1952) separated eight components in an *Aspergillus oryzae* extract, with varying degrees of specificity towards different substrates, whereas a *Stachybotrys atra* preparation hydrolysed *p*-nitrophenyl β-glucoside but not *o*-nitrophenyl β-glucoside or cellobiose (Jermyn, 1955). Differences in relative cellobiase and salicinase activities in preparations from different sources were observed by Grassmann, Zechmeister, Tóth & Stadler (1933) and Reese & Levinson (1952). Barnett, Ingram & Swain (1956) studied the β-glucosidase activity of different yeasts and found that all split aesculin, but many would not split any one of arbutin, salicin or cellobiose. In the present investigation *o*-nitrophenyl β-glucoside, cellobiose and salicin have been used in a comparative study of β-glucosidase activity from cell-free extracts of mixed micro-organisms from sheep rumen.

EXPERIMENTAL

Materials. *o*-Nitrophenyl β-D-glucoside was kindly provided by Dr J. Conchie. Cellobiose (shown to be chromatographically pure) and glucono-1:4-lactone were obtained from L. Light and Co. Ltd., salicin from Hopkins and Williams Ltd., glucose oxidase from Sigma Chemical Co., and peroxidase from C. F. Boehringer und Soehne.

β-Glucosidase preparations. These were produced by butanol extraction of sheep-rumen micro-organisms centrifuged at 1500 *g*, as described previously (Festenstein, 1958).

Methods of enzyme assay

o-Nitrophenyl β-glucosidase. The procedure of Conchie (1954) was modified as follows: enzyme (0·5 ml.), substrate and 0·8 ml. of 0·1M-citric acid–0·2M-Na_2HPO_4 buffer, pH 5·8 (McIlvaine, 1921), in a total volume of 4 ml. (final pH 6·1) were incubated for 1 hr. and the reaction was stopped by adding 4 ml. of 0·25M-glycine–Na_2CO_3 buffer, pH 10·0 (pH of mixture, 10·0). (This buffer was prepared by mixing 32·6 g. of glycine and 25·4 g. of NaCl in 800 ml. of water, adjusting to pH 9·6 with 50% NaOH, adding 400 ml. of 1·25M-Na_2CO_3 and diluting to 2 l.)

Cellobiase. Conditions for assay and reducing-sugar determination (by the cuprimetric method only) were as described previously (Festenstein, 1958). Glucose was measured specifically by glucose oxidase (Huggett & Nixon, 1957); solutions were deproteinized as for reducing-sugar determination or alternatively the reaction was stopped by placing them in a boiling-water bath for 10 min. (the enzyme blank in either case was negligible). Cellobiose itself gave a small blank (2–3% of the value expected for an equal weight of glucose).

Salicinase. Incubation mixtures (1 ml.) were used for reducing-sugar determination without previous deproteinization, since the activity of the enzyme preparations permitted the use of very dilute solutions. Saligenin was determined by the method of Kerr, Graham & Levvy (1948) for phenol, modified as follows, to eliminate deproteinization: 1·5 ml. of reagent (Folin–Ciocalteu Reagent, British Drug Houses Ltd., diluted 1:4·2) was added, followed by 5 ml. of N-Na_2CO_3 before incubation for colour development; Ilford no. 608 filters (wavelength of peak transmission, 680 mμ) were used in the Spekker absorptiometer (Levvy & Marsh, 1952). Saligenin (British Drug Houses Ltd.), twice recrystallized from water, was used as a standard. Glucose did not interfere with colour production, nor did saligenin affect the reducing value of glucose.

RESULTS

Activities of enzyme preparations. Illustrative examples of enzyme activities are shown in Table 1.

In salicinase studies where both saligenin and reducing sugar were estimated, it was found that the ratio saligenin:reducing sugar increased with increasing substrate concentration. For salicin concentrations between 0·7 mM and 20 mM the reducing substances liberated increased only slightly, up to 2·8 mM, and then decreased, whereas the saligenin liberated increased throughout the range; the saligenin:reducing sugar ratio rose by over one-third between 2·8 mM and 20 mM. Reducing-sugar determination by the cuprimetric method was not possible for nitrophenyl glucosidase assays, since substantial amounts of substrate were hydrolysed during heating with copper reagent.

The glucose oxidase method was apparently unsuitable for glucose determination in the presence of liberated saligenin since a purple colour appeared, probably due to reaction of saligenin with oxidized *o*-dianisidine; phenol produced a similar colour. *o*-Nitrophenol liberated would likewise interfere, but in this case on account of its own colour quite apart from any possibility of further reaction.

Hydrolysis of substrate mixtures. The use of a chromogenic substrate enables other substances to be tested as possible competing substrates (Levvy & Marsh, 1954). Conchie (1954) established that 10 mM-cellobiose produced 30% inhibition of rumen *o*-nitrophenyl β-glucosidase; a detailed study of the nature of the inhibition showed that cellobiose acts chiefly as a competitive inhibitor.

Table 1. *Hydrolysis of different β-glucosides by rumen-enzyme preparations*

Enzyme activity was measured in 0·02M-citric acid–0·04M-Na_2HPO_4 buffer, pH 6·1, or 0·02M-citric acid–NaOH buffer, pH 6·0 (for salicinase), with the following substrates: 5·8 mM-cellobiose, 2·5 mM-*o*-nitrophenyl β-glucoside, 1·4 mM-salicin. All values are expressed as μg. of substance liberated/ml. of reaction mixture/hr. at 37° and are within the range of linear hydrolysis of substrate with time. The original enzyme preparations were diluted five times in the cellobiase assay mixture and 400 times for assay with the other two substrates.

Sheep no.	Cellobiase (μg. of glucose, estimated as reducing sugar)	*o*-Nitrophenyl β-glucosidase (μg. of *o*-nitrophenol)	Salicinase (μg. of saligenin)
44	800	19	24
91	360	8·6	8
107	213	10	—

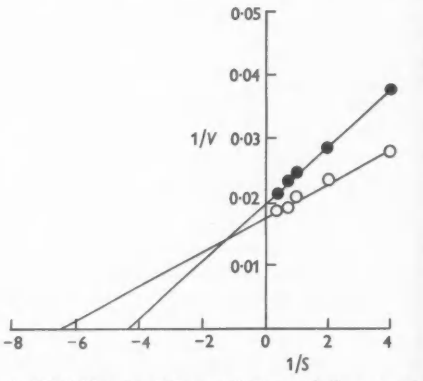

Fig. 1. Inhibition by 5·8 mM-cellobiose of the enzymic hydrolysis of *o*-nitrophenyl β-glucoside. Results for both the inhibited (●) and uninhibited (○) reactions are plotted according to Woolf (1932) and Lineweaver & Burk (1934): *V*, velocity of the reaction = μg. of *o*-nitrophenol liberated/75 min.; *S*, substrate concn. = mM-*o*-nitrophenyl β-glucoside. Kinetic constants obtained here are K_m, 0·16 mM; K_i (competitive component), 13 mM; K_i (non-competitive component), 50 mM. K_m determined directly for cellobiase was 0·9 mM.

but there is slight non-competitive inhibition (Fig. 1). This finding was consistently reproduced with eight different enzyme preparations, obtained from three sheep, the concentrations of cellobiose varying from 3·6 mM to 19 mM. Salicin (0·7 mM and 10 mM) was shown to be essentially a competitive inhibitor only, preparations from four different sheep being used. Glucose (11 mM) and gluconolactone (0·02 mM, 0·1 mM) were also competitive inhibitors, the latter substance behaving as established previously (Conchie, 1954).

Salicin could be used as a chromogenic substrate in the same way as nitrophenyl glucoside by estimating the saligenin liberated; cellobiose acted as a competitive inhibitor at a concentration of 3·6 mM, but a definite non-competitive component appeared for 19 mM. Gluconolactone (0·02 mM) was a competitive inhibitor.

DISCUSSION

The use with β-glucosidases of substrates such as nitrophenyl glucoside and salicin, where aglycone liberated is measured, provides more accurate results than measurement of reducing substances liberated. However, a study of both parameters is also useful as is observed from the increasing saligenin:reducing sugar ratio with increasing substrate concentration; this type of phenomenon may be due to transferase activity, as has been observed by Jermyn & Thomas (1953).

The specific glucose oxidase method has valuable potentialities in cellobiase studies, particularly since deproteinization is apparently unnecessary and the substrate blank is very small compared with that obtained by the best reducing methods. A complication is the coupling of the oxidized dye with saligenin in salicinase studies.

The results obtained with the substrate mixtures suggest that the substrates investigated act chiefly as competitors for the same site, or sites, of the enzyme molecule. The non-competitive component for the hydrolysis of nitrophenyl glucoside in the presence of cellobiose and also that obtained with 19 mM-cellobiose as inhibitor of salicinase, suggests a different or additional site of combination. Hofstee (1955), using almond extracts, showed cellobiose, glucose and salicin to be mainly non-competitive inhibitors of the hydrolysis of salicylic acid β-glucoside and claims that the finding may have a bearing on the theory of Pigman & Goepp (1948), that the glucose and aglycone moieties of the substrate combine with different groups on the enzyme molecule.

SUMMARY

1. β-Glucosidase activity of cell-free extracts of micro-organisms from sheep rumen has been measured with *o*-nitrophenyl β-glucoside, cellobiose and salicin as substrates.

2. Saligenin liberated from salicin has been shown to increase with substrate concentration even when the reducing substances liberated reach a constant value and decrease.

3. Cellobiose acts chiefly as a competitive inhibitor of the hydrolysis of *o*-nitrophenyl β-glucoside; salicin, glucose and gluconolactone are essentially competitive inhibitors.

Thanks are due to Dr G. A. Levvy for his criticism and encouragement, to Dr J. Conchie for the *o*-nitrophenyl β-glucoside, to Professor A. St G. Huggett for further details of the glucose oxidase method, to Dr G. Halliwell for providing the samples of rumen liquor and to Mrs E. F. Robertson for technical assistance.

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Microdetermination of the Fatty Acids in Blood Serum

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Herb & Riemenschneider (1953) and Evans, Waldron, Oleksyshyn & Riemenschneider (1956) have described methods for the spectrophotometric determination of polyethenoid fatty acids in blood depending upon the change in optical density at five different wavelengths due to isomerization with potassium hydroxide in ethylene glycol at 180°. In the present paper this technique and that of O'Connell, Lipscomb & Daubert (1952) have been modified, making it possible to perform duplicate determinations on 2 ml. of serum. The method of van de Kamer, Pikaar, Bolssens-Frankena, Couvée-Ploeg & van Ginkel (1955) for the determination of higher saturated fatty acids has also been modified to permit such analyses with only 4 ml. of serum. This method depends on reverse-phase chromatography on a rubber column (Boldingh, 1953). A determination of the total fatty acid content can easily be included, which allows the calculation of the total unsaturated fatty acid content. The amount of total unsaturated and total polyethenoid fatty acids being known, the monoethenoid fatty acids can be calculated by subtraction.

METHODS

Reagents

Potassium hydroxide (21%, *w/w*) *in ethylene glycol.* This was prepared according to Herb & Riemenschneider (1952). After heating to 190°, it is preferable to cool the ethylene glycol to room temperature before adding the KOH and to flush well with nitrogen before heating again to 190°. The reagent, prepared in this way, and stored under nitrogen at approx. −20°, remained essentially colourless for at least one year.

Absolute methanol. This was prepared with aluminium amalgam (Pestemer, 1951).

Purified nitrogen. Purest cylinder nitrogen is washed twice with 0·1M-vanadyl sulphate according to Meites & Meites (1948), and afterwards twice with 20% NaOH and twice with water.

Rubber for chromatography. This is purified by percolation in a column (90 cm. × 4 cm.) with 3 l. of a mixture of acetone and 25% (w/w) HCl (9:1) for 9 hr. instead of boiling with acetone–HCl (van de Kamer *et al.* 1955).

Thymol blue solution. 0·1% in 96% ethanol, not neutralized.

Other reagents. See van de Kamer *et al.* (1955).

As the amounts of fat which are handled are very small, it is important to use thoroughly cleaned solvents and glassware.

Preparation of extract of fatty acids from serum

Serum (2 ml.) is added dropwise and under agitation, to a 100 ml. volumetric flask, with ground-glass stopper, containing about 60 ml. of ethanol–ether (3:1, v/v; Bloor, 1914). After adding some carborundum chips, the solution is boiled for 2 min., cooled to room temperature, filled up to the mark and filtered through a fat-free filter. A volume (80 ml.) of the filtrate is pipetted into a 100 ml. Erlenmeyer flask, and 2 ml. of N-sodium ethoxide, 0·1 ml. of 0·1% ethanolic quinol solution (Wilson & Hansen, 1935) and some carborundum chips are added. The solvent is distilled off completely on a water bath, the last traces being blown off with nitrogen. The soap is transferred to a 100 ml. separating funnel (stopcock and stopper lubricated with glycerol) with 36 ml. of 75% (v/v) ethanol. The solution is shaken three times with 10 ml. of light petroleum (b.p. 40–60°), the combined extracts are shaken once with 8 ml. of 75% ethanol and the latter is added to the soap solution. The soap solution is transferred to the separating funnel with 36 ml. of water, and after acidifying with 2 ml. of 4N-HCl, extracted three times with 10 ml. of light petroleum. The combined extract is dried with anhydrous Na_2SO_4, filtered and made up to 50 ml. with light petroleum.

Determination of polyethenoid fatty acids

Isomerization. Fatty acid solution (15 ml.) in light petroleum is transferred in duplicate to the reaction tubes (Fig. 1) and some carborundum chips are added. The solvent is evaporated on a water bath under nitrogen. The fatty acids are concentrated in the bottom of the tube by washing down with light petroleum. To the residue, 0·5 ml. of KOH in ethylene glycol is added by means of a measuring pipette, connected with rubber tubing to a syringe. Nitrogen is flushed over for 15 min. and the tubes are placed, at intervals of 1 min., in the bath, kept at 180° ± 0·3; each tube is shaken after 30, 60 and 90 sec. After being heated for exactly 15 min., the tubes are cooled under tap water and the reaction mixture is diluted with methanol to 10 ml. in volumetric flasks with ground-glass stoppers. A blank of 0·5 ml. of reagent is run through the whole procedure. Optical densities are read against the blank on a Beckman DU spectrophotometer at 268, 315, 346 and 374 mμ, with 1 cm. quartz cells. A methanol dilution (1:5) is made for the density reading at 233 mμ.

For the density readings before isomerization, 15 ml. of extract in light petroleum is evaporated under nitrogen in a reaction tube, overheating being avoided (the optical

densities are not influenced by this treatment). The residue is dissolved in 10 ml. of methanol and optical densities are read at the wavelengths mentioned above.

Calculation. From the increases in density (i.e. the difference between the density before and after isomerization), determined at the appropriate wavelengths, and the total fatty acid concentration (determined separately) the values of k_{233}, k_{268}, k_{315}, k_{346} and k_{374} are calculated. These are the increases in density that could be obtained if the density measurements were carried out at a concentration of 1 g. of total blood fatty acids/l. of methanol. The content of each of the polyethenoid fatty acids (as a percentage of the total blood fatty acids) may then be calculated from the following equations (Herb & Riemenschneider, 1953; Hammond & Lundberg, 1953):

Linoleic acid (%) = $1{\cdot}093\ k_{233} - 0{\cdot}573\ k_{268} - 0{\cdot}259\ k_{315} - 0{\cdot}033\ k_{346} - 0{\cdot}279\ k_{374}$

Linolenic acid (%) = $1{\cdot}105\ k_{268} - 0{\cdot}879\ k_{315} + 0{\cdot}190\ k_{346} - 1{\cdot}245\ k_{374}$

Arachidonic acid (%) = $1{\cdot}650\ k_{315} - 1{\cdot}667\ k_{346} - 0{\cdot}079\ k_{374}$

Pentaenoic acid (%) = $1{\cdot}449\ k_{346} - 1{\cdot}351\ k_{374}$

Hexaenoic acid (%) = $3{\cdot}413\ k_{374}$

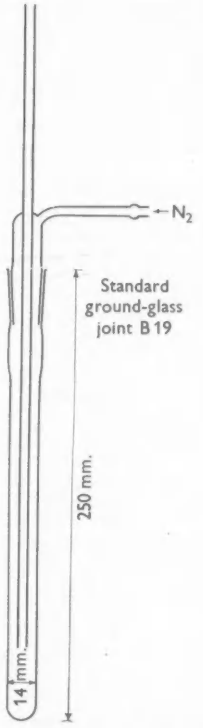

Fig. 1. Tube of Pyrex glass for the isomerization of the polyethenoid fatty acids.

Effect of the saponification on the polyethenoid fatty acids

As it might be expected that the saponification causes an undesirable premature isomerization of the polyethenoid fatty acids, due to the heating with concentrated alkali, the absorption spectra of the serum extracts after single, double and no saponification were determined.

To resaponify, the completely dry soap is dissolved in 2 ml. of N-sodium ethoxide and 0·1 ml. of 0·1% ethanolic quinol solution, the mixture homogenized thoroughly while warming in the water bath and the solvent distilled off again under nitrogen. The soap is extracted with light petroleum in the described manner.

To prepare a serum extract without saponification, the solvent of the Bloor's extract is distilled off under nitrogen and the residue is extracted several times with light petroleum (b.p. 40–60°).

For the determination of the absorption spectra, the solution in light petroleum is evaporated under nitrogen, the residue is dissolved in 10 ml. of methanol and the optical densities are read at intervals of 2 mμ. The absorption spectra are shown in Fig. 2.

The absorption curve before isomerization is lowest after single saponification and shows weakly the characteristic peaks of conjugated polyethenoid fatty acids. As these peaks are increased after double saponification and are

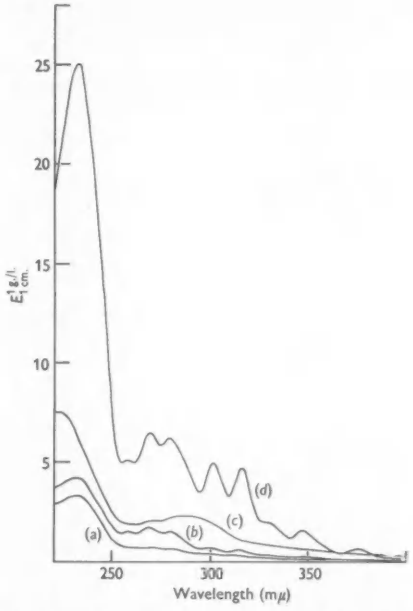

Fig. 2. Absorption spectra of the fat of pooled serum *B* before and after isomerization: (*a*), (*b*) and (*c*) after a single, double and no saponification respectively and without isomerization; (*d*) after a single saponification and isomerization.

completely absent without saponification, it appears that the conjugation is caused by the saponification. Consequently, no conjugated polyethenoid fatty acids are present in human serum.

Determination of total and saturated fatty acids

The method for the determination of the saturated fatty acids (van de Kamer *et al.* 1955) is modified as follows.

Duplicate portions (2 ml.) of serum are extracted and saponified as described and the light petroleum extracts are titrated, after addition of 20 ml. of ethanol, under nitrogen with approx. 4 mN-sodium ethoxide. (Alternatively, one fatty acid extract in light petroleum can be prepared, taking appropriate quantities of serum and reagents, serving for the determination of both the polyethenoid fatty acids and the total and saturated fatty acids.) After titration, which yields the total fatty acid content, taking an average molecular weight for the fatty acids of 277, the two solutions are combined and the solvent is distilled off completely. The soap is dissolved in 27 ml. of water and 1 ml. of 6N-KOH, and 2 ml. of 4·7% $KMnO_4$ solution is added. The solution is mixed and allowed to stand for 1 hr. After adding 2 ml. of 6N-H_2SO_4 and solid $NaHSO_3$ to remove the excess of $KMnO_4$, the solution is extracted three times with 10 ml. of light petroleum (b.p. 40–60°). The combined extracts are dried with anhydrous Na_2SO_4 and the solvent is distilled off. The residue is concentrated in the bottom of a test tube and can easily be added to the rubber column in three transfers by means of 1 ml. of acetone–neutral arachis oil mixture (4:1) with a Pasteur pipette. During the treatment with $KMnO_4$, no saturated fatty acids are attacked; however, the acids C_8 and C_{10} are not quantitatively extracted from the mixture.

The iodine value was measured by a modification of the method of Wijs (1898). To the soap solution, remaining after a determination of the total fatty acid content (or to a portion of the fatty acid extract in light petroleum, corresponding to 2 ml. of serum), 0·1 ml. of 0·1% ethanolic quinol solution is added and the solvent is distilled off completely. To the residue, 10 ml. of 0·02N-Wijs's solution is added and well mixed. After standing for 1 hr. in the dark, 50 ml. of water and 10 ml. of 0·05N-KI solution are added and the mixture is titrated with 0·02N-sodium thiosulphate. A blank is treated in the same manner.

Table 1. *Increases in optical density ($E^{1\,g./l.}_{1\,cm.}$) produced by isomerization of cod-liver oil by different methods*

For the density readings before isomerization, 200 mg. of cod-liver oil was dissolved in 50 ml. of 2:2:4-trimethylpentane. From this solution a 1:5 dilution was made for the reading at 233 mμ.

(*a*) 70–80 mg. of cod-liver oil isomerized according to Herb & Riemenschneider (1952). The reaction mixture was diluted with methanol to 100 ml. and from this solution a 1:20 dilution was made with methanol.

(*b*) 40 mg. of lauryl alcohol, containing approx. 4 mg. of cod-liver oil, isomerized according to Herb & Riemenschneider (1953). The reaction mixture is diluted to 100 ml. with methanol.

(*c*) 10 ml. of light petroleum (b.p. 40–60°), containing approx. 0·4 mg. of cod-liver oil, is evaporated and the residue isomerized as described under Methods. The reaction mixture is diluted to 10 ml. with methanol.

(*d*) Same as (*c*), but with 15·5 min. reaction time, instead of 15 min.

Wavelength (mμ)	233	268	315	346	374
Macro method (*a*)	15·9±0·9	15·7±1·3	14·6±0·3	10·0±0·7	3·5±0·3
Micro method (*b*)	13·6±0·8	12·7±0·6	14·5±0·7	11·2±0·6	4·0±0·3
Modified micro method (*c*)	13·7±0·4	12·5±0·2	13·8±0·3	10·6±0·4	3·8±0·1
Modified micro method with 15·5 min. reaction time (*d*)	13·5±0·3	12·3±0·3	13·5±0·3	10·4±0·3	3·6±0·2

Table 2. *Determination in replicate of the fatty acids in two pooled sera*

Figures given are mean values, the number of determinations being given in parentheses.

	Pooled serum *A*		Pooled serum *B*	
	(mg. %)	(% of total fatty acids)	(mg. %)	(% of total fatty acids)
Linoleic acid	66·3±1·1 (12)	19·4	67·4±1·2 (12)	19·3
Linolenic acid	6·7±1·0 (12)	2·0	5·1±0·5 (12)	1·5
Arachidonic acid	17·4±0·9 (12)	5·1	17·7±0·4 (12)	5·1
Pentaenoic acid	4·3±0·5 (12)	1·3	3·3±0·5 (12)	0·9
Hexaenoic acid	6·3±0·1 (12)	1·8	5·8±0·6 (12)	1·7
Lauric acid	4·5±1·1 (3)	1·3	3·8±1·0 (12)	1·1
Myristic acid	7·6±0·7 (3)	2·2	7·7±2·4 (12)	2·2
Palmitic acid	75·3±2·8 (3)	22·0	78·2±3·4 (12)	22·5
Stearic acid	20·0±3·0 (3)	5·9	22·3±2·0 (12)	6·4
Total fatty acids	342 ±4·1 (40)	100	348 ±6·7 (36)	100
Total saturated fatty acids	107 ±2·4 (3)	31·3	112 ±5·3 (12)	32·2
Total polyethenoid fatty acids	101 ±2·5 (12)	29·6	99 ±2·0 (12)	28·5
Monoethenoid fatty acids	134	39·2	137	39·4
Iodine value {Found	—	—	104·8±2·1 (12)	—
Iodine value {Calc.	106·2	—	103·0	—
Mean mol. wt. of fatty acids, calc.	276·8	—	276·7	—

RESULTS

Comparison of different methods

The validity of the new method has been tested by carrying out comparative determinations both with the macro and the micro method of Herb & Riemenschneider (1952, 1953). For this purpose we used a sample of cod-liver oil, since this oil contains polyethenoid fatty acids with two to six double bonds. The results of the determinations in cod-liver oil are shown in Table 1. As fish oils are known to contain polyethenoid fatty acids to which the normal calculation cannot be applied (Herb & Riemenschneider, 1953), the results are given in optical densities only. The modified method and the micro method of Herb & Riemenschneider agree very well, whereas the macro method shows somewhat higher optical densities at 233 and 268 mμ and lower optical densities at 346 and 374 mμ. Statistical treatment cannot be applied because of the mutual dependance of the measured optical densities at different wavelengths.

Accuracy and normal values

To test the accuracy of the new method, two samples of pooled sera (*A* and *B*) collected from several hundred patients were each analysed 12 times. The results, including the calculated values for the monoethenoid fatty acids, the found and calculated iodine values and the calculated mean molecular weights of the fatty acids, are given in Table 2.

The standard deviations are identical for both sera and do not vary much for small and large quantities. The calculated iodine value for pooled serum *B* is in good agreement with the experimentally determined value. The calculated mean molecular weight of the fatty acids corresponds to the values in the literature, i.e. 277·2 (Smith & Kik, 1933); 281 (Wilson & Hansen, 1935); 277 (Pagé & Michaud, 1951). In Table 3 the values are presented for ten fasted, normal males. The mean composition for these ten persons is identical with that of the two samples of pooled sera.

DISCUSSION

From the experiments with cod-liver oil (Table 1) it follows that the results with the new method are identical with the original micro method of Herb & Riemenschneider (1953). Although an important point of difference is that the reaction tubes with reagent are not warmed before addition of the sample, the experiments under (*d*) indicate that this effect can be neglected. The explanation for the deviation between the two methods of Herb & Riemenschneider remains obscure, nevertheless it seems justifiable to use their constants for the new method.

Table 3. *Determination of the fatty acids in serum of fasted, normal male subjects*

Results are expressed as percentage of the total fatty acid content, except the total fatty acid value itself, which is expressed in mg. %. From the mean values, shown in the last column, the calculated mean molecular weight and iodine value for the fatty acids are 276·9 and 106·3 respectively. In subject 3 plasma was used for the determination.

Subject	1	2	3	4	5	6	7	8	9	10	Mean
Total fatty acids	270	635	218	372	315	389	394	275	407	316	359±115
Total saturated fatty acids	28·1	31·6	26·4	31·4	32·8	31·0	32·2	32·0	31·3	31·1	30·8±2·0
Total polyethenoid fatty acids	35·4	23·9	34·4	32·8	31·0	31·3	24·9	30·6	24·9	27·1	29·6±4·2
Monoethenoid fatty acids	36·6	44·6	39·2	35·8	36·2	37·8	42·9	37·4	44·0	41·5	39·6±3·4
Linoleic acid	26·3	15·4	23·1	20·2	17·9	22·5	15·2	19·3	15·8	19·4	19·5±3·7
Linolenic acid	0·6	1·9	1·1	3·0	2·4	2·5	3·0	2·5	2·1	2·1	2·1±0·8
Arachidonic acid	4·3	3·9	5·7	6·9	6·1	4·0	4·7	6·7	4·8	3·6	5·1±1·2
Pentaenoic acid	1·6	1·2	1·7	1·1	1·3	1·1	0·7	1·2	1·0	0·9	1·2±0·3
Hexaenoic acid	2·6	1·5	2·9	1·4	3·3	1·2	1·2	0·8	1·3	1·2	1·7±0·9
Lauric acid	0·8	0·8	0·3	1·0	1·2	0·5	0·6	1·5	1·2	1·0	0·9±0·4
Myristic acid	2·3	3·1	0·9	2·4	2·8	3·2	2·5	2·8	2·6	2·0	2·5±0·7
Palmitic acid	18·2	22·4	18·2	20·8	22·7	21·4	22·4	20·7	21·0	20·2	20·8±1·6
Stearic acid	6·8	5·3	7·0	7·0	6·1	5·9	6·7	7·1	6·4	8·0	6·6±0·7

When the method is applied to serum fat, it is necessary to saponify the fat beforehand, since it contains components which are difficult to saponify and which may therefore give erroneous results. Front & Daubert (1945) showed that unsaponified cholesteryl linoleate gives too low results, possibly because of the insolubility of the ester in the reagent. However, the saponification causes a premature, undesirable conjugation of the polyethenoid fatty acids (Fig. 2), but this proves to be very small, as the results of the analysis of the polyethenoid fatty acids after single and double saponification are identical. In contrast, the results of the analysis without saponification show higher values for pentaenoic and hexaenoic acid, perhaps because of a changed absorption spectrum of the impurities during the treatment with hot alkali. Comparison of the curves (*a*) and (*c*) of Fig. 2 makes it clear that the saponification removes most of the impurities of the serum fat. Moreover, other difficulties are encountered when isomerizing the serum fat without saponification. When a Bloor's extract is evaporated, an insoluble precipitate is formed. After extraction of the residue with light petroleum, approx. 5% of the total fatty acids, including polyethenoid fatty acids, remain undissolved. This is demonstrated by saponifying the extracted residue and isomerizing the fatty acids obtained by the described method, the absorption curve showing clearly the peaks of conjugated polyethenoid fatty acids.

The finding that no conjugated polyethenoid fatty acids occur in human serum (Fig. 2, curve *c*) contradicts the findings of Zirm & Schauenstein (1952), who state that conjugated dienoic, trienoic and tetraenoic acids are present in normal serum. However, these authors did not demonstrate characteristic absorption peaks.

With the micro method of Herb & Riemenschneider (1953) milligram quantities of fat can be determined, weighing of very small quantities being avoided by diluting the sample with lauryl alcohol. However, the amount of reagent (5 ml.) requires a rather large volume of methanol to transfer the reaction mixture quantitatively, which results in low optical-density readings. In the present method 0·5 ml. of reagent is used, making optical density readings possible in 10 ml. of solution. The use of measured fractions of a light-petroleum extract avoids also in this method the weighing of small amounts of fat.

By means of the alkaline-isomerization technique, only the systems of two to six isolated double bonds are determined. However, *iso*-acids are believed to occur only in traces in blood fat, so that with the described method the true composition will be closely approximated. Additional data about the identity and the extinction coefficients of the pentaenoic and hexaenoic acids in blood serum would be desirable, but as these acids occur only in small quantities the influence on the calculation of the content of other acids will be negligible.

The modifications in the method of van de Kamer *et al.* (1955) allow an accurate determination of small amounts of saturated fatty acids; traces of saturated fatty acids with an uneven carbon chain or with a branched chain, possibly present in blood fat, cannot be detected by this method. With the new purification of the rubber, acid products from the rubber during analysis are reduced to a minimum. In previous work (van de Kamer *et al.*, 1955; Pikaar, 1955) neutralized thymol blue indicator was used for the titration of the 20 ml. fractions, resulting in too low recoveries for the acids C_8–C_{14}. However, when unneutralized indicator is used, good recoveries are obtained for all acids, also for small quantities. For a good result, blanks have to be taken from a control column.

Examination of the Tables 2 and 3 shows that 40% of the serum fatty acids are monoethenoid, 30% are polyethenoid, linoleic and arachidonic acid being the greatest components. The remaining 30% consists of saturated fatty acids, of which about three-quarters is palmitic acid, and the rest mainly stearic acid with traces of myristic and lauric acids. Table 3 shows that although the total serum-fatty acid content of fasted subjects shows a wide variance, nevertheless the composition of the fatty acid mixture is rather constant.

Comparison of our results with the data of Evans *et al.* (1956) for the plasma polyethenoid fatty acids on pooled blood from two persons shows a good agreement; separate data, given for five persons, are somewhat different. Their values for average oleic acid (21·1%) and total saturated fatty acids (41·9%), calculated from the iodine value, differ rather from our values (40 and 30% respectively).

SUMMARY

1. A method is described for the determination of the different fatty acids in blood serum.

2. The polyethenoid fatty acids are determined by a combination of the method of Herb & Riemenschneider (1952, 1953) and that of O'Connell *et al.* (1952), in 2 ml. of serum. The results are essentially the same as those obtained with the original method of Herb & Riemenschneider. A saponification is necessary if the method is applied to serum fat.

3. The saturated fatty acids are determined, after removal of the unsaturated fatty acids, by a modification of the method of Boldingh (1953), 4 ml. of serum being required. A determination of the total fatty acid content is included.

4. The monoethenoid fatty acids are calculated by difference.

5. Replicate analyses of two samples of pooled human sera indicate a good reproducibility. Values for normal human blood serum are given.

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The Effect of Soluble Silica on the Metabolism of Rat-Tissue Preparations

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The free silicas (amorphous, quartz, cristobalite and tridymite), in a finely divided form, are strongly fibrogenic in the tissues of man and experimental animals (see, for example, Gardner, 1938; King, Mohanty, Harrison & Nagelschmidt, 1953). Solubilities of about 14 mg. of silica/100 ml. in Ringer solution and blood plasma have been observed with the free silicas. Silicates (e.g. felspar, mica, sericite) at the same particle size are weakly fibrogenic, if at all, and their silica solubilities are comparatively low, 1 mg. of silica/100 ml. or less. This correlation has been taken as evidence that soluble silica is the toxic agent responsible for the pathogenicity of silica dusts (King, 1950). Other evidence in support of the solubility theory of silicosis is assembled and critically discussed by King (1947, 1950).

The present study concerns the effect of soluble silica on the metabolism of rat-tissue preparations. Many possibilities must be considered for the mode of action of a tissue poison. For example, it might inhibit the activity of one or more enzymes (King, Schmidt, Roman & Kind, 1956, have observed considerable inhibitions of acetylcholine esterase, succinic dehydrogenase, liver esterase and hyaluronidase with 0·025 M-silicic acid), in which case it would need first to penetrate to the site of activity, or it might change the permeability of cell membranes or protoplasm towards certain substrates. With such considerations in mind the effect of soluble silica on aspects of metabolism of rat-tissue slices has been investigated. The effect on the respiration of tissue homogenates is also described.

Preliminary reports of this work have been published (Rowsell & Leonard, 1957*a*, *b*).

EXPERIMENTAL

Soluble-silica preparations. Finely ground Madagascar quartz, 99·8% SiO_2, was supplied by the Safety in Mines Research Establishment, Sheffield; 0·75 g. was intimately mixed with 4·5 g. of anhydrous Na_2CO_3 in a platinum crucible. The mixture was heated until a clear melt was obtained, and extracted with water, with heat to facilitate solution, and made up to 100 ml.

A column (45 cm. long, 2·5 cm. in diameter) of the cation-exchange resin Zeo-Karb 225 (The Permutit Co. Ltd., London) was prepared in the H^+ ion form. After standing with 2 N-HCl for 30 min. it was washed with water until neutralization of 100 ml. of effluent required less than 1 ml. of 0·02 N-NaOH, with methyl orange as indicator. The sodium silicate solution was poured on the column and silicic acid sols were obtained when the effluent was in the range pH 3·3–3·7. The sols were gassed with N_2 or O_2 to

remove dissolved CO_2 and stored in polythene containers. Visual flame comparisons showed that the concentration of sodium in the sols was less than 0·004%. Total silica was estimated in each sol gravimetrically; usually 6·0–6·5 mg. of SiO_2/ml. was found.

Silica sols contain mono- and di-silicic acids which react rapidly with molybdic acid, higher polymers of silicic acid which react very slowly and colloidal silica which does not react. It is therefore possible to measure the concentration of mono- and di-silicic acids in the presence of higher polymers (Alexander, 1953, 1954).

Some sols were analysed for total silica by the colorimetric method of Case (1944), as modified by Paterson & Wheatley (1955), and for molybdate-reactive silica (King, Pash & Weedon, 1954). Measured portions of these sols were neutralized (bromothymol blue as indicator) with a few drops of 0·1N-NaOH, diluted with homogenate medium or tissue-slice saline, and incubated at 37° in polythene vessels (see Fig. 1). Samples were removed at different times and the concentration of molybdate-reactive silica was estimated by the method of King *et al.* (1954) so that the phosphate present would make no contribution to the development of blue colour. Optical densities of the solutions were measured at 800 mμ. Silica standards were prepared from sodium silicofluoride according to King (1939). A stable level of about 200 μg. of molybdate-reactive silica/ml. was always attained. This was independent of the original level of molybdate-reactive and polymerized silica in the undiluted sol, where the total silica exceeded 200 μg./ml. (Fig. 1).

The concentration of molybdate-reactive silica in freshly prepared undiluted sols was in the range 1000–1500 μg./ml. This diminished over a period of days to the stable level of 200 μg./ml.

Pre-incubations with soluble silica in metabolic experiments. In all the metabolic studies soluble silica was pre-incubated with saline and substrates for 100 min. at 37°, to ensure that this stable level of molybdate-reactive silica had been reached, before homogenate or slices were added. Similar pre-incubations were included in control experiments, with water replacing silica sol. Sols (6 mg. of SiO_2/ml.) were diluted 1:5 for the metabolic experiments to give 1·2 mg. of SiO_2/ml. (0·02M-total SiO_2). From the results presented in Fig. 1 it is evident that the 0·02M-total SiO_2 included 200 μg./ml. or 0·003M-molybdate-reactive silica, and 0·017M-polymerized silica.

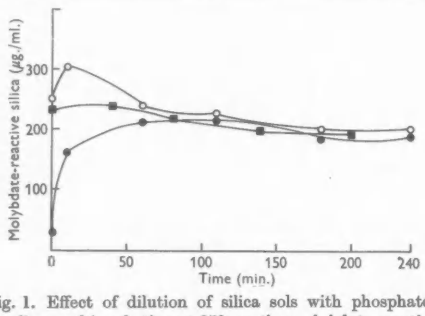

Fig. 1. Effect of dilution of silica sols with phosphate–saline, and incubation at 37°, on the molybdate-reactive silica. The undiluted sols had a total SiO_2 concentration of 7 mg./ml.; 1 vol. of sol was mixed with saline: ●, 19 vol.; ■, 4 vol.; ○, 1·5 vol.

Siliceous dusts. Finely divided tridymite, quartz, mica and felspar, graded by differential centrifuging to include only particles in the range 0·5–2·0 μ, were prepared at the Safety in Mines Research Establishment, Sheffield.

Tissue preparations and incubation conditions

Laboratory-stock albino rats have been used throughout. After stunning they were killed by severing the blood vessels of the neck.

Homogenates. Livers were removed immediately into ice-cold medium (13 vol. of 1·15% KCl, 2 vol. of 0·1M-sodium phosphate buffer, pH 7·3, 0·5 vol. of 3·82% $MgSO_4,7H_2O$). Liver samples (2 g.) were homogenized with 8·5 ml. of medium in a cold steel Potter–Elvehjem (1936) homogenizer and strained through gauze to remove fibrous material.

Warburg vessels contained 0·5 ml. of homogenate, 0·2 ml. of 0·2M-substrate, 0·2 ml. of diphosphopyridine nucleotide (DPN) (10 mg./ml. when added), 0·8 ml. of SiO_2 (6 mg./ml.) or of water, and medium to a total of 4 ml.; NaOH (0·2 ml. of 10N-solution) and filter paper were added to the centre wells. The gas phase was O_2.

In some experiments substrates were added to the side bulbs and mixed with the main vessel contents after equilibration in the water bath. However, incubation of the tissue preparation at 37° for 8 min. in the absence of substrate gave considerable diminution in the respiration rates, observed after mixing, with or without silica. In most experiments therefore the cold homogenate was added to the medium already containing substrate, and the first manometric readings were noted when the vessels had been in the bath 7–8 min.

Slices. For tissue-slice experiments the phosphate saline normally used (a modification of medium III, Krebs, 1950) was made up as follows:

0·9% NaCl, 103 vol.
1·15% KCl, 4 vol.
2·11% KH_2PO_4, 1 vol.
3·82% $MgSO_4,7H_2O$, 1 vol.
1·3% $NaHCO_3$, 3 vol.
} Solution *A*.

2·8 vol. of sodium phosphate, 0·1M, pH 7·3, and 2·6 vol. of 0·11M-$CaCl_2$, were added to 100 vol. of solution *A* whilst gassing with O_2.

Organs were removed into ice-cold saline. Slices were cut with a Stadie-Riggs (1944) slicer and collected in saline. They were weighed on a torsion balance after adherent saline had been drained off by touching the slices against a clean Petri dish.

Respiration studies. About 100 mg. of liver or spleen, and 50 mg. of kidney or lung, were taken for individual incubations at 37°. Warburg vessels contained 0·2 ml. of 0·2M-substrate when added, 0·8 ml. of SiO_2 (6 mg./ml.) or of water, and phosphate saline to 4 ml. The gas phase was O_2.

Acetoacetate synthesis. The same conditions applied in studying the effect of soluble silica on the synthesis of acetoacetate in liver slices (about 130 mg.) from 0·02M-acetate. Rats starved for 17 hr. were employed in these experiments. After the incubations (120 min.) slices were removed and acetoacetate in the medium was estimated manometrically, with aniline citrate to catalyse decarboxylation (Edson, 1935).

Glycolysis. Slices of liver (about 100 mg.), spleen (50 mg.) and lung (80 mg.) suspended in 4 ml. of phosphate–saline (with or without silica) containing 0·01 M-glucose were incubated in 25 ml. conical flasks accommodated in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill., U.S.A.) at 37°, with air as the gas phase. Lactic acid accumulating in the medium was estimated by the method of Barker & Summerson (1941), as modified by LePage (1949). Irregular values with standards (18 μg. of lactic acid as zinc lactate) were first obtained. Consistent results followed when the amounts of reagents added were increased to 0·1 ml. of 4% $CuSO_4,5H_2O$ and 0·15 ml. of 1·5% *p*-hydroxydiphenyl in 0·5% NaOH.

Urea synthesis. For studies on urea synthesis the Warburg vessels contained 3 ml. of bicarbonate medium (Krebs & Henseleit, 1932) with or without silica, 0·01 M-NH_4Cl and liver slices (about 200 mg.). In some experiments DL-lactate was also present, 0·007 M with each stereoisomer. Incubations were for 90 min. at 37° with O_2+CO_2 (95:5) as gas phase. Urea accumulating in the medium was estimated manometrically with jack-bean-meal urease (Krebs, 1942).

Silica at the concentration used in the slice experiments had no effect on the estimation of acetoacetate [prepared from ethyl acetoacetate (Ceresole, 1882)], lactic acid or urea.

Substrates. DPN (95% pure) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other substrates were of analytical grade. Organic acids and NH_4Cl were neutralized to pH 7·3 with NaOH before use.

RESULTS

Effect of silica on the respiration of rat-liver homogenates

Aged silica sols (more than 7 days after preparation) invariably inhibited O_2 consumption (30–70% measured on the first 15 min. of observations) in rat-liver homogenates with succinate, fumarate, L-malate, β-hydroxybutyrate or DL-lactate as substrate. DPN was added with all substrates except succinate.

Some fresh sols gave strikingly different results. With succinate, β-hydroxybutyrate or DL-lactate as substrate the rates of O_2 uptake were not significantly affected. With fumarate and L-malate there was little difference in the initial respiration rate, but whereas this diminished in the controls the rate with silica was maintained and the total O_2 consumption was thereby much increased (more than twofold in some experiments) (Fig. 2). This effect was observed only with fumarate and L-malate as substrates and was given with six out of nine fresh sols examined (Fig. 2). The other fresh sols all gave 30–50% inhibition with all substrates tested.

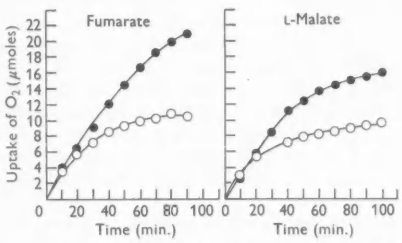

Fig. 2. Effect of soluble silica on the respiration of rat-liver homogenates with 0·01 M-fumarate or L-malate. DPN was present in each incubation. ○, Controls; ●, with 0·02M-SiO_2.

When initial tests with a fresh silica sol failed to show this maintenance of respiration with fumarate and L-malate, then repeated experiments with the same sol and other rat-liver preparations were also negative in this respect. Apparently the inconsistency was to be attributed to variation in the nature of the silica sols, perhaps in the molecular size of the silicic acid polymers.

In all experiments the tissue homogenate was precipitated within 2–3 min. of its addition to medium containing 0·02 M-silica. No effect was observed on respiration with succinate, fumarate or β-hydroxybutyrate when the silica concentration was 0·003 M, 180 μg./ml. (i.e. with only molybdate-reactive silica).

The effect of 25 mg. of tridymite or quartz dust has been examined on the respiration of rat-liver homogenates with fumarate or L-malate as substrate under the same conditions. The maintenance of respiration obtained with fresh silica sols was never observed. In some experiments tridymite and quartz gave 20% inhibition of respiration, but in parallel incubations greater inhibition (40–60%) was observed with 25 mg. of mica or felspar (non-pathogenic dusts). It was therefore concluded that the inhibitions observed with tridymite and quartz had no direct bearing on the problem of silicosis.

Effect of silica on the metabolism of rat-liver slices. Soluble silica (0·02 M) was without effect on the respiration of liver-, spleen-, kidney- or lung-tissue slices. For all tissues investigated, observations on O_2 consumption have been continued for periods up to 3 hr. Respiration rates diminished, but not more so in the presence of silica than in the controls. Where the addition of a suitable substrate produced an increased respiration (e.g. L-glutamate with kidney and succinate with spleen and lung slices), the same increase was observed where silica was present. This is taken to signify that the silica did not prevent the penetration into the tissue cells of the substrates concerned.

Soluble silica had no effect on the formation of urea from ammonia and CO_2, or on the formation of acetoacetate from acetate by rat-liver slices.

There was always a slight increase in the aerobic rate of lactate production from glucose in the presence of soluble silica, with slices of liver, spleen

Table 1. *Effect of soluble silica on aerobic glycolysis in rat-tissue slices*

Glucose (0·01 M) was present in each incubation. In each experiment individual slices were cut into two approximately equal pieces. One half was used as a control, the other with SiO_2 medium.

			Lactic acid formation (μmole/g. of wet slice/hr.)	
	Tissue		Control	With 0·02 M-SiO_2
(i)	Liver	(*a*)	7·0	8·5
		(*b*)	8·3	9·3
		(*c*)	6·0	7·5
	Spleen	(*d*)	12·6	14·4
		(*e*)	13·5	14·4
		(*f*)	16·2	17·4
(ii)	Liver	(*g*)	5·7	6·3
		(*h*)	5·5	6·5
	Lung	(*i*)	8·8	9·6
		(*j*)	10·4	12·4

and lung (Table 1). The same results were obtained in the various tissue-slice investigations with fresh and aged sols. Sols have been used from 1 to 11 days after preparation.

DISCUSSION

In the present investigation a far higher concentration of soluble silica has been employed than is ever likely to occur *in vivo*. Even under the most favourable conditions, i.e. maintained dispersion of fine dust with continuous shaking, the rate at which silica dissolves is very slow, e.g. 20 μg. of molybdate-reactive silica/ml./hr. from 6·2 g. of quartz or tridymite in 100 ml. of Ringer solution (Paterson & Wheatley, 1955), and that dissolving from silica dust introduced into the tissues (e.g. lungs) of experimental animals is quickly excreted in the urine (King, 1950). Until experimental evidence is obtained indicating localized accumulations *in vivo* it must be assumed that these do not occur.

Furthermore, there is no evidence that silicic acid coming into solution from solid particles of silica is every highly polymerized. Paterson & Wheatley (1955) found that tridymite, quartz, cristobalite and fused silica did not give any colloidal polymerized silica on treatment with Ringer solution. They attribute positive findings in this respect (Clelland, Cummings & Ritchie, 1952) to incomplete sedimentation of very fine particles of crystalline solids, and to the employment of analytical techniques which would not differentiate between finely divided solid silica in suspension and silica in true colloidal solution. There is need to extend this work, with discriminating techniques to investigate the amount and form of silica dissolving from dusts in media more closely approximating in composition to those found *in vivo*.

Provisionally it must be assumed that only low polymers of silica (molybdate-reactive) dissolve from silica dusts, and that these do not accumulate *in vivo* in sufficient concentration to condense to form highly polymerized silicic acid. Effects obtained with highly polymerized silicic acid must be regarded as having doubtful relevance to the aetiology of silicosis.

Nevertheless, it was decided to employ as high a concentration of soluble silica as could be conveniently introduced, in the metabolic investigations, so that any effect silica might have would be more readily observed. The maximum attainable level of molybdate-reactive silica (about 200 μg./ml.) has been employed, together with highly polymerized silica, to make a total of 1200 μg. of silica/ml. (0·02 M). These two forms of silica have been simultaneously tested.

Fumarate, L-malate, β-hydroxybutyrate and DL-lactate all require DPN for their oxidation in animal-tissue preparations. The observation that colloidal silica maintains respiration only with fumarate and L-malate (Fig. 2) makes it unlikely that the silica could have produced this effect by maintaining the DPN level, e.g. by inhibiting the liver enzyme catalysing the hydrolytic breakdown of DPN (Handler & Klein, 1942).

When fumarate and L-malate are oxidized enzymically oxaloacetate accumulates and inhibits the reaction. The possibility that silica in some way facilitates the removal of oxaloacetate may be considered. A demonstration of a specific interaction between soluble silica and oxaloacetate (a key metabolite) would be of great interest.

Inconsistency in the results obtained has hindered the development of this line of work. It seems that there is some critical unstable state in which fresh silica sols behave differently from aged sols. Moreover, not all fresh sols prolonged respiration with fumarate and L-malate. A standardized procedure has been followed in the preparation of silica sols (see Experimental section), but there are variables (e.g. the room temperature at which sols were prepared and kept), possibly of importance, which have not been taken into account. Before this investigation can be profitably pursued, it will be necessary to find conditions for preparation of silica sol where this biochemical effect can be consistently observed.

Soluble silica had no effect on the respiration of slices of rat tissues. Liver, spleen and lung were included in this study since extensive fibrosis in response to silica dusts has been observed in these organs in many investigations, in experimental animals or in human post-mortem material (e.g. in rat lung, King *et al.* 1953; in rabbit liver, Gardner, 1938; in human spleen, Belt, 1939).

Aspects of metabolism have been studied which involve the integrated action of many individual

enzymes: glucolysis, urea synthesis (see, for example, Ratner, 1951) and acetoacetate synthesis (e.g. Kennedy & Lehninger, 1952). Thus in each experiment the effect of silica on many metabolic processes, some of vital importance to the cell, was being observed. Silica had no effect on urea or acetoacetate formation in rat-liver slices, and slightly increased the aerobic glycolysis rate in liver, spleen and lung slices.

These results indicate that soluble silica did not diminish the permeability of the cells to a variety of substances (e.g. oxygen, carbon dioxide, ammonia, lactate, glucose, urea, acetate, acetoacetate) and that a considerable number of enzymes catalysing a great diversity of reactions (including oxidative phosphorylation) are immune to the influence of the silica penetrating the cells.

An investigation is being made on the extent of penetration of soluble silica into tissue slices. Preliminary results indicate that there is no barrier to the diffusion of molybdate-reactive silica into the cells of rat-liver and -kidney slices. The concentration attained in the total tissue water is that of the surrounding medium (Rowsell & Leonard, 1957*b*).

Marks, Mason & Nagelschmidt (1956) have observed a toxic effect of silica dusts on leucocytes *in vitro*, and they suggest that injury to the phagocytic cells by dust is the stimulus for formation of fibrous tissue. Leucocytes ingest silica dusts in the animal body, and this is the only situation *in vivo* where silica dusts make direct contact with cell cytoplasm. It is conceivable that polymerized silica comes into solution in this particular environment but evidence is lacking. James & Marks (1956) found that soluble silica (0·005M, a lower concentration than has been employed in the present work) severely depressed the respiration of guinea-pig leucocytes. (This total silica concentration presumably included 0·003M-molybdate-reactive silica and 0·002M-polymerized silica; see Experimental section.) They found no inhibition of leucocyte respiration when only molybdate-reactive silica was present.

The results of the present study suggest that soluble silica is not a toxic substance affecting the tissues generally. However, it may be that more complex metabolic activities, e.g. synthesis of protein or nucleic acid, are inhibited by soluble silica or it may be that it can affect specifically only one cell type, the leucocyte.

SUMMARY

1. Soluble silica (0·02M), which includes 0·003M-mono- and -di-silicic acids, and higher polymers (0·017M), prolonged the respiration of rat-liver homogenates with fumarate and L-malate. Highly polymerized silicic acid was responsible for this effect.

2. Soluble silica had no effect on the respiration of slices of rat liver, kidney, spleen or lung, nor on urea or acetoacetate formation in liver slices.

3. A slight increase in aerobic glycolysis was observed with slices of liver, spleen and lung in the presence of soluble silica.

4. Implications of these negative findings with tissue slices for the solubility theory of silicosis are discussed.

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Some Characteristics of the Enzymic Carboxyl Activation of Methionine

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Hoagland (1955) reported the presence in rat liver of enzymes which catalyse the formation of adenylates from amino acids and adenosine triphosphate (ATP), and suggested the following scheme (PP represents pyrophosphate) for the reaction:

$$\text{Enzyme} + \text{ATP} + \text{HO}_2\text{C}\cdot\text{R}\cdot\text{NH}_2 \rightleftarrows \text{Enzyme–AMP–CO}\cdot\text{R}\cdot\text{NH}_2 + \text{PP} + \text{H}_2\text{O}.$$

The binding of the carboxyl group of the amino acids to the adenosine monophosphate (AMP) is considered as an activation of the carboxyl group. This activation was confirmed by the fact that in the presence of hydroxylamine it was possible to demonstrate the presence of the corresponding hydroxamates of the amino acids, according to the reaction:

$$\text{Enzyme–AMP–CO}\cdot\text{R}\cdot\text{NH}_2 + \text{NH}_2\cdot\text{OH} \rightleftarrows \text{Enzyme} + \text{AMP} + \text{H}_2\text{N}\cdot\text{R}\cdot\text{CO}\cdot\text{NH}\cdot\text{OH}.$$

Later, Hoagland, Keller & Zamecnik (1956) extended those observations and tested the specificity of different enzyme preparations towards the various amino acids. They were able to purify, by ammonium sulphate fractionation of the liver extract, an enzyme which was specific for the activation of the carboxyl group of methionine. Davie, Konigsberger & Lipmann (1956), starting with pancreas, obtained an enzyme preparation which was specific for tryptophan. Later they studied the specificity of this enzyme towards tryptophan analogues with a view to determining the role of these enzymes in protein synthesis (Sharon & Lipmann, 1957).

Although the role of peptides in protein synthesis is not yet clear it is of interest to determine whether an enzyme preparation which is specific for the activation of an amino acid activates the amino acid when it is in a peptide linkage. The specificity of the methionine-activating enzyme towards methionine peptides has therefore been studied. The action of various metabolic inhibitors on the enzyme activity has also been studied to determine whether the inhibition of protein synthesis by such compounds can be related to the inhibition of these enzymes. A preliminary report of some aspects of these results has been published (Rendi, 1956).

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MATERIALS AND METHODS

Enzyme preparation. Wistar albino rats weighing 150–200 g. were used. The animals were killed by bleeding and the liver was removed as rapidly as possible. The methionine-activating enzyme was extracted with 0·05 M-KCl, and the extract was fractionated with ammonium sulphate between 35 and 45% of saturation as described by Hoagland *et al.* (1956).

Fractionation of the liver. Rat liver was homogenized with 2 vol. of 0·25 M-sucrose in a Teflon (polytetrafluoroethylene) Potter–Elvehjem-type homogenizer and the suspension was centrifuged at 18 000 *g* in an International refrigerated centrifuge for 1 hr. at +1°. The pellet was resuspended and homogenized with 1 vol. of 0·25 M-sucrose and centrifuged in a similar manner. The combined supernatants were dialyzed for 20 hr. at +2° against 0·05 M-KCl, and the dialyzed supernatant was fractionated with ammonium sulphate. The pellet, containing nuclei, mitochondria and microsomes, was homogenized with 3 vol. of 0·05 M-KCl, and centrifuged for 1 hr. at 18 000 *g*, and the supernatant treated with ammonium sulphate.

Measurement of activation. The reaction mixture contained, in 1 ml. of 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol buffer (pH 7·6), 1·2 m-mole of hydroxylamine (free base), 10 μmoles of Mg^{2+} ion, 10 μmoles of adenosine triphosphate (ATP) (Sigma Chemical Co., St Louis, Mo., U.S.A.), brought to pH 7·6 with NaOH, and an appropriate amount of the enzyme [between 1·5 and 3 mg. of protein determined by the method of Gornall, Bardawill & David (1949) in the final 1 ml.] and the different substances to be tested. The mixture was incubated at 37° for 2 hr., and the quantity of hydroxamate was determined by the method of Hoagland *et al.* (1956). This method depends on the formation of a red colour when $FeCl_3$ in acid solution is added to hydroxamates. In a typical experiment the amount of hydroxamate formed in the presence of 12 μmoles of methionine is between 1·2 and 2·5 μmoles. Because of this great variation in the amount of hydroxamate formed, the data are reported as a percentage of the amount formed in the presence of methionine.

Hydroxylamine was prepared from hydroxylamine hydrochloride dissolved in methanol by neutralizing with saturated NaOH in methanol. The precipitated NaCl was filtered off, and the free base was precipitated by adding to the filtrate an equal amount of ether. The precipitate was washed with methanol–ether mixture and ether.

Table 1. *Intracellular localization of the methionine-activating enzyme*

Results are expressed as a percentage of the colour intensity with methionine as substrate with the enzyme preparation from the soluble fraction of the cytoplasm. The particulate fraction contained nuclei, mitochondria and microsomes. The three experiments were carried out with different tissue preparations, and data reported are the mean value of the experiment ± the standard deviation.

No. of experiments	Intracellular fraction	Hydroxamate formed: Without methionine	Hydroxamate formed: With 12 μmoles of methionine
3	A 0·05 M-KCl extract of the particulate fraction	10±2	12±2
3	Soluble fraction of cytoplasm	30±3	100

Table 2. *Enzymic formation of methionine hydroxamate in the presence of nucleic acids*

Results are expressed as a percentage of the colour intensity with methionine as substrate. In each experiment 0·5 mg. of nucleic acid in the final 1 ml. volume was added. The data represent the mean value ± the standard deviation.

No. of experiments	Substances added	Hydroxamate formed: Without methionine	Hydroxamate formed: With 12 μmoles of methionine
9	None	30±2	100
5	RNA	28±3	68±4
5	RNA hydrolysate	35±3	82±5
3	DNA	34±2	99±3

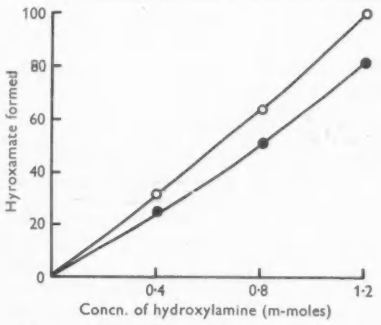

Fig. 1. Enzymic formation of methionine hydroxamate in the presence of an enzymic hydrolysate of RNA with different concentrations of hydroxylamine. The hydroxamate formed is expressed as a percentage of the colour intensity observed with methionine alone at the highest hydroxylamine concentration. ○, Without RNA hydrolysate; ●, with RNA hydrolysate (0·5 mg. in the final 1 ml. volume).

Ribonucleic acid hydrolysate. A portion (200 mg.) of thymus ribonucleic acid (RNA) (Bayer, Leverkusen, Germany) was dissolved in 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol buffer (pH 6·6) with 50 μg. of crystalline ribonuclease (Sigma Chemical Co.), and the solution was dialysed for 24 hr. at room temperature. The ribose concentration in the diffusate was determined colorimetrically (Dische & Schwartz, 1937) and the solution was diluted to the required concentration.

Other substances. All amino acids and peptides were DL. Protamine was a salmine sulphate preparation obtained from British Drug Houses Ltd., and yeast deoxyribonucleic acid was obtained from Bayer. The metabolic inhibitors were obtained from Merck, Darmstadt, Germany.

RESULTS

Intracellular localization of the activating enzyme. The fractions purified from the particulate fractions or from the soluble cytoplasm of rat liver were brought to equal protein concentrations (between 2·8 and 3·0 mg. of protein in the final volume of 1 ml.) and their activating capacity was tested with methionine.

Table 1 shows that only the enzyme preparation from the soluble fraction of cytoplasm catalysed the formation of hydroxamate in the presence of methionine, whereas the preparation from the particulate fractions of liver was inactive.

Action of nucleic acids on formation of hydroxamate. Hoagland & Zamecnik (1957) suggested that in a cell-free system the amino acids after their activation become associated with RNA molecules. Therefore the action of nucleic acids, and of an enzymic hydrolysate of RNA, on the enzymic formation of methionine hydroxamate was studied. In *Staphylococcus* (according to Gale, 1955) the enzymic hydrolysate of RNA stimulates protein synthesis, as does the undegraded RNA.

Table 2 shows that the presence of RNA or of RNA hydrolysate inhibits hydroxamate formation, whereas DNA is ineffective. The inhibitory effect of the RNA hydrolysate is smaller than that observed with the unhydrolysed RNA. The curves presented in Fig. 1 show that the hydroxylamine concentration does not influence the inhibition exerted by the RNA hydrolysate, which is always approximately 20%.

Activation of peptides. As shown in Table 3, two different peptides containing methionine with its carboxyl group in a terminal position are activated by the purified enzyme, the hydroxamate formation in the presence of alanylmethionine being lower than that of the others. The determinations of hydroxamate formation with different quantities of substrates (Fig. 2) show that the rate

of formation of hydroxamate is less for alanylmethionine at all concentrations and is 60% of that formed in the presence of methionine. Although experimental data on the intensity of the colour reaction between $FeCl_3$ and peptide hydroxamates have not been reported, it is known that the colour intensities in the $FeCl_3$ reaction produced by various amino acid hydroxamates differ up to 20% (Schweet, 1955). The value obtained for alanylmethionine hydroxamate formation is so much below that of methionine that it can hardly be accounted for by a difference in the production of colour intensity.

Effects of inhibitors of protein synthesis. Among the different inhibitors of protein synthesis three groups of substances were studied: (i) amino acid analogues (ethionine 12 mM); (ii) the substances which interfere with nucleic acid metabolism (ribonuclease and protamine); (iii) inhibitors of oxidative phosphorylation and glycolysis (NaN_3, 2:4-dinitrophenol, NaF, all at 25 mM).

Table 3. *Hydroxamate formed by the purified enzyme with different substrates*

Results are expressed as a percentage of the colour intensity with methionine as substrate. In each experiment 12 μmoles of substrate in the final 1 ml. volume were added. The data are reported as mean value $\pm$ standard deviation.

No. of experiments	Substrate	Hydroxamate formed
10	None	30$\pm$2
12	Methionine	100
6	Glycylmethionine	100$\pm$4
6	Alanylmethionine	60$\pm$3

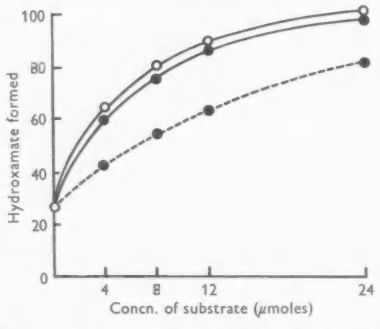

Fig. 2. Effect of different concentrations of substrates on the enzymic formation of hydroxamates. The hydroxamate formed is expressed as a percentage of the colour intensity observed for the higher methionine concentration. ○—○, Methionine; ●—●, glycylmethionine; ●- - -●, alanylmethionine.

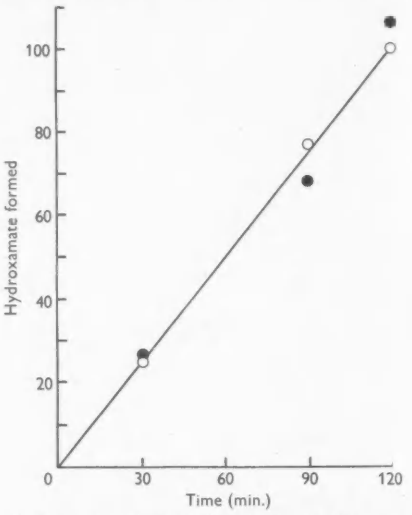

Fig. 3. Enzymic activation of methionine in the presence of ethionine (12 μmoles of each amino acid in the final 1 ml. volume). The hydroxamate formed is expressed as a percentage of the colour intensity observed for methionine alone at the end of the experiment. ○, Without ethionine; ●, with ethionine.

Table 4. *Enzymic formation of methionine hydroxamate in the presence of ribonuclease and protamine*

Results are expressed as a percentage of the colour intensity with methionine as substrate. In each experiment 50 μg. of ribonuclease and 0·5 mg. of protamine in the final 1 ml. volume were added. The data are reported as mean value $\pm$ standard deviation.

		Hydroxamate formed	
No. of experiments	Substance added	Without methionine	With 12 μmoles of methionine
8	None	30$\pm$2	100
5	Ribonuclease	38$\pm$3	107$\pm$4
4	Protamine	42$\pm$4	110$\pm$5

Table 5. *Enzymic formation of methionine hydroxamate in the presence of inhibitors of oxidative phosphorylation and glycolysis*

Results are expressed as a percentage of the colour intensity with methionine as substrate. In each experiment 12 μmoles of methionine and 25 μmoles of the metabolic inhibitors were added. The data are reported as mean value $\pm$ standard deviation.

No. of experiments	Substance added	Hydroxamate formed
8	Methionine	100
4	Methionine and NaF	99$\pm$4
4	Methionine and NaN_3	100$\pm$5
4	Methionine and dinitrophenol	101$\pm$4

The failure of ethionine to inhibit the formation of methionine hydroxamate during incubation for 2 hr. is shown in Fig. 3. It has also been observed that ethionine is not activated by the methionine-activating enzyme and also that ethionine does not inhibit the formation of hydroxamates of methionine peptides.

The action of ribonuclease and protamine upon methionine hydroxamate formation is summarized in Table 4. It can be seen that in the presence of these compounds there is an increase in hydroxamate formation, but that such an augmentation is also found in the control experiments with ribonuclease or protamine but no amino acids present. Therefore it might be assumed that such compounds act as substrates for the enzyme preparations and in any case do not inhibit hydroxamate formation.

Table 5 shows the action of inhibitors of protein synthesis, which interfere with oxidative phosphorylation and glycolysis. NaF, NaN_3 and dinitrophenol did not affect the hydroxamate formation. It was not possible to test the action of $\alpha\alpha'$-dipyridyl and monoiodoacetate, because the first compound reacts with $FeCl_3$ and with the second a non-enzymic reaction with hydroxylamine occurs which yields a compound that reacts with the ferric chloride reagent.

DISCUSSION

The results presented here show that no methionine-activating enzyme can be extracted from the particulate fraction of rat liver with 0·05 M-KCl. This observation is consistent with the data reported by Zamecnik & Keller (1954) and those reported by Hoagland *et al.* (1956), who found that soluble cytoplasm is necessary for incorporation of amino acids into microsomal proteins, and that the soluble fraction of cytoplasm can be replaced in this case by a protein fraction of the cytoplasm containing activating enzymes.

The idea that RNA plays a role in protein synthesis is now widely accepted. The hypothesis of Zamecnik, Keller, Hoagland & Littlefield (1956) was recently confirmed by Hoagland & Zamecnik (1957) by showing that radioactive amino acids are transferred after their activation to a soluble RNA fraction. The present results, showing that RNA inhibits the formation of hydroxamate, can also be taken as an indication that RNA participates in a step of protein synthesis related to activation of amino acids, although the RNA used, being extracted from yeast, is not specific for liver-activating enzymes, and more experiments should be made with RNA prepared from liver.

It was previously shown (Rendi, 1955) that the hydroxamate formation in the presence of an amino acid mixture catalysed by the soluble fraction of rat liver described by Hoagland (1955) was the same if glycine in the mixture was replaced by diglycine or triglycine, and it was suggested that the carboxyl group of diglycine and triglycine, like that of glycine, was activated by liver. In the present paper it is shown that peptides containing methionine with its carboxyl group in the terminal position are also activated by the enzyme preparation which activates the methionine carboxyl group only. These results differ from those reported by Davie *et al.* (1956), working with the tryptophan-activating enzyme. This enzyme did not activate either *N*-acetyltryptophan or glycyltryptophan, in contrast with glycylmethionine and the methionine-activating enzyme. It should, however, be mentioned that the tryptophan-activating enzyme is much purer than the enzyme preparation that was used in this investigation (Hoagland *et al.* 1956), and the possibility that this preparation also contains the enzymes specific for the activation of methionine peptides cannot be excluded. It is, however, unlikely that a different enzyme exists for the specific activation of each peptide; it is more probable that not all peptides can be activated. The activation of peptides reported here supports the possibility that such compounds could be incorporated into proteins without previous hydrolysis to free amino acids.

The findings of a lack in inhibition of the methionine-activating enzyme by sodium fluoride, sodium azide and dinitrophenol is in agreement with the supposition that such compounds inhibit amino acid incorporation into proteins by interfering with the mechanisms which provide the energy for the formation of peptide bonds.

The observation reported above, that ribonuclease and protamine do not inhibit the activity of the methionine-activating enzyme, supports the hypothesis that RNA has no direct action on the initial step of protein synthesis. A finding which points in the same direction is that of Davie *et al.* (1956), who found that their purified tryptophan-activating enzyme contained nucleotides, chiefly guanosine monophosphate, but that it did not lose any of its activity when deprived of this.

Another characteristic which distinguishes the methionine- from the tryptophan-activating enzyme is its behaviour in the presence of amino acid analogues. Davie *et al.* (1956) and later Sharon & Lipmann (1957) observed that the tryptophan-activating enzyme can also activate 2-azatryptophan, 7-azatryptophan and 5-fluorotryptophan but not 5- or 6-methyltryptophan or 2-aza-6-methyltryptophan. They suggest that this enzyme activates only those analogues which are incorporated into the proteins (i.e. the first three). In contrast, in the present study, ethionine, the characteristic

analogue of methionine, did not form the corresponding hydroxamate in the presence of the methionine-activating enzyme and did not inhibit methionine activation. The inability of the methionine-activating enzyme to use ethionine as a substrate is in contrast with the observed incorporation of radioactive ethionine into liver proteins (Levine & Tarver, 1951) and into protein in Ehrlich ascites cells (Rabinovitz, Olson & Greenberg, 1957). However, Rabinovitz *et al.* (1957) observed that less ethionine is incorporated into proteins than methionine, and this difference could account for the inability to demonstrate ethionine activation in isolated enzyme preparations.

SUMMARY

1. Rat-liver homogenate prepared in 0·25 M-sucrose was separated into particulate and supernatant fractions. The presence of an enzyme activating the carboxyl group of methionine could be demonstrated in the supernatant, but no such activity was found in a 0·05 M-potassium chloride extract of the particulate fraction.

2. The enzyme was inhibited by yeast ribonucleic acid, and by an enzymic hydrolysate of ribonucleic acid, by 20–30 %, but not by thymus deoxyribonucleic acid.

3. Glycylmethionine and alanylmethionine were activated by the enzyme preparation, but alanylmethionine activation was lower than that of methionine or glycylmethionine.

4. Inhibitors of protein synthesis (12 mM-ethionine, ribonuclease, protamine, 25 mM-sodium fluoride, 25 mM-sodium azide, 25 mM-dinitrophenol) failed to inhibit the enzyme activity.

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Studies on Plant Flavokinase

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As riboflavin is a component of several enzymes concerned with respiration, cells must be capable of synthesizing the active forms of the vitamin, namely both flavin mononucleotide and flavin-adenine dinucleotide. The earlier observations (Hubner & Verzar, 1939; Pulner & Verzar, 1939), that phosphorylation of riboflavin could take place by means of inorganic phosphate in preparations of intestinal mucosa, seemed unlikely in view of the energetics of the reaction. A conclusive demonstration of the phosphorylation of riboflavin by adenosine triphosphate was made by Kearney & Englard (1951), using a purified enzyme preparation from yeast. The only reports in the literature pertain to its isolation and purification from different yeasts (Kearney & Englard, 1951; Giri & Krishnaswamy, 1956). Though the occurrence of riboflavin in plants is well known, little is known about the mechanism of synthesis of flavin nucleotides in plants. It was observed that synthesis of flavin mononucleotide took place in plant extracts when they were incubated under the same conditions as those used with yeasts (Giri, Krishnaswamy & Appaji Rao, 1957). The properties of this plant enzyme have been investigated and are described in this paper.

MATERIALS AND METHODS

Substrates and reagents. Riboflavin and flavin mononucleotide (FMN) were products of Hoffman La-Roche and Nutrition Biochemicals Corp. respectively. Adenosine

Table 1. *Progress of purification of flavokinase from* Phaseolus radiatus

For details of stages of purification see text. Reaction mixtures were as follows: 0·2 ml. of riboflavin, 0·1 mM; 0·1 ml. of ATP, mM; 0·1 ml. of NaF, 0·1 M; 0·7 ml. of 0·1 M-veronal–HCl buffer (pH 8·6); 0·8 ml. of enzyme preparation from the various stages and 0·1 ml. of $MgSO_4$, 0·3 mM. Total volume 2 ml. Incubated at 55° for 1 hr.

Stage of purification	Volume (ml.)	Protein (mg.)	Total activity (units)	Specific activity (units/mg. of protein)
I	165	15 820	6198	0·3908
II	180	4 842	8246	1·7030
III	60	820	6726	8·2040
IV	130	416	5981	14·5100
V	40	105	3144	29·9300

triphosphate (ATP) used included preparations made according to LePage (1949), of purity 60–65%, and one purchased from California Foundation for Biochemical Research of purity 95%. Some differences in reactions were noted with samples of ATP of different purity but these differences were only quantitative in nature. All other chemicals used were of a reagent grade and the purest currently available.

Preparation and purification of enzyme. Finely powdered green gram (*Phaseolus radiatus*) was used as the source of the enzyme, and various procedures were tried to obtain extracts with maximum activity. Extraction of the powder at a temperature of 0–5° with water was found to be more efficient than extraction with buffer solutions or with water at 25° and 37°. The procedure finally adopted, which was very similar to that used by Kearney & Englard (1951) for yeast flavokinase, was as follows.

Finely powdered material was extracted with water (100 g./300 ml.) for 60 min. at 0–5° by periodic shaking. The extract obtained by centrifuging at 1000 *g* (165 ml., stage I, Table 1) was dialysed in the cold against water for 18–20 hr. and the precipitate which appeared was centrifuged at 2500 *g*. To the opalescent supernatant (180 ml., stage II) ammonium sulphate to 0·40 saturation was added and precipitate obtained by centrifuging at 5000 *g* was dissolved in 50 ml. of water and dialysed for 18 hr. against water at 0–5° (60 ml., stage III). An equal volume of alumina-C_γ gel (15 mg./ml.) was added to the dialysed solution and kept for 20 min. in the cold, and centrifuged at 1000 *g*. This procedure removed much inactive protein; the precipitate was eluted three times with 10 ml. portions of 0·02 M-sodium phosphate buffer (pH 7·2) and recombined with the supernatant (130 ml., stage IV). This was again fractionated with ammonium sulphate to 0·40 saturation; the precipitate obtained by centrifuging at 5000 *g* was dissolved in 30 ml. of 0·01 M-veronal–HCl buffer (pH 8·6) and the solution dialysed for 18 hr. against water in the cold (40 ml., stage V).

A 75-fold purification of the enzyme was achieved with a recovery of about 38%. It is likely that crude extracts contain certain inhibitory factors which are eliminated on dialysis, since the activity increased on dialysis.

Enzyme assay. During the assay and analysis, tubes (10 cm. × 1 cm.) in which reactions were carried out were protected from light. The reaction mixtures, unless otherwise stated, consisted of 0·2 ml. of riboflavin solution, 0·1 mM; 0·1 ml. of ATP solution, mM; 0·1 ml. of NaF solution, 0·1 M; 0·1 ml. of $MgSO_4$ solution, 0·3 mM; 0·7 ml. of 0·1 M-veronal–HCl buffer (pH 8·6) and 0·8 ml. of enzyme preparation; total volume, 2 ml. They were incubated at 55° and at specified intervals, usually after 60 min., the reaction was arrested by the addition of 0·8 ml. of 17·5% (w/v) trichloroacetic acid. The tubes were kept in a boiling-water bath for 5 min. to hydrolyse traces of flavin-adenine dinucleotide (FAD) formed. The solutions were cooled, centrifuged and suitable portions of the supernatant were analysed for FMN formed by the circular-paper-chromatographic method of Giri & Krishnaswamy (1956), with butanol–acetic acid–water (4:1:5, v/v) as the solvent system. The procedure briefly consisted in locating FMN on the chromatograms under u.v. light, eluting the paper strips in glass-distilled water and measuring the fluorescence in a Klett fluorimeter. Appropriate blanks and controls were always run. For preparative purposes, the technique of Giri (1954, 1955) was used. Absorption spectra were determined with a Beckman Spectrophotometer, Model DU.

Riboflavin concentrations in germinating seedlings were determined by the method of Snell & Strong (1939). Protein was determined by the Biuret method. One unit of enzyme activity is defined as the amount that synthesizes 1 μm-mole of FMN at pH 8·6, temperature 55°, in 60 min.

RESULTS

Identification of flavin mononucleotide formed by enzymic synthesis. A reaction mixture (40 ml.), containing the substrates, buffer, etc., as described was incubated for 18 hr. at 37°. The reaction was stopped with 16 ml. of 17·5% (w/v) trichloroacetic acid and the mixture was kept in a boiling-water bath for 5 min. The supernatant was subjected to preparative circular-paper chromatography (Giri, 1954, 1955). The isolated product showed absorption maxima at 264–266, 372–374 and 446 mμ and was chromatographically identical with an authentic sample of FMN. On enzymic hydrolysis by phosphatases [0·2 ml. of 0·1 mM-FMN; 0·8 ml. of 0·1 M-veronal–HCl buffer (pH 8·6) and 1 ml. of enzyme preparation from green gram], as well as acid hydrolysis with N-H_2SO_4 at 100° for 1 hr., phosphate and riboflavin were detected as degradation products.

Reverse reaction. FMN (0·2 ml., 0·1 mM); 0·1 ml. of $MgSO_4$, 0·3 mM; 0·8 ml. veronal–HCl buffer (pH 8·6); and 0·9 ml. of the purest enzyme preparation (total volume 2 ml.) were incubated at

37° for 2 hr. Reaction was arrested by the addition of 0·8 ml. of 17·5% (w/v) trichloroacetic acid. Portions of supernatant were analysed chromatographically with butanol–acetic acid–water (4:1:5, v/v) as the solvent system. No riboflavin could be detected on the chromatograms. Kearney & Englard (1951) have reported that the synthesis of FMN from riboflavin and ATP was virtually an irreversible reaction.

Optimum temperature for activity of enzyme. In preliminary experiments reactions were carried out at 37°. However, it was found later that the enzyme showed maximum activity at 55° (Fig. 1).

The plant flavokinase was different from the yeast flavokinase in that it had a much higher temperature for optimum activity. Whereas the enzyme was totally inactivated when incubated alone at 60°, much activity (90%) was retained when it was incubated with its substrates (riboflavin 0·1 mM, ATP 1·0 mM). This would explain to a certain extent the high temperature of optimum activity.

Progress curve of the reaction. This is shown in Fig. 2; 2 ml. reaction mixtures were set up under standard conditions as already stated. At different intervals, they were analysed for FMN. Separate sets of tubes were used for each time interval.

pH–*Activity curve.* This is shown in Fig. 3. Variation of the initial rate of reaction as a function of pH was determined by carrying out experiments,

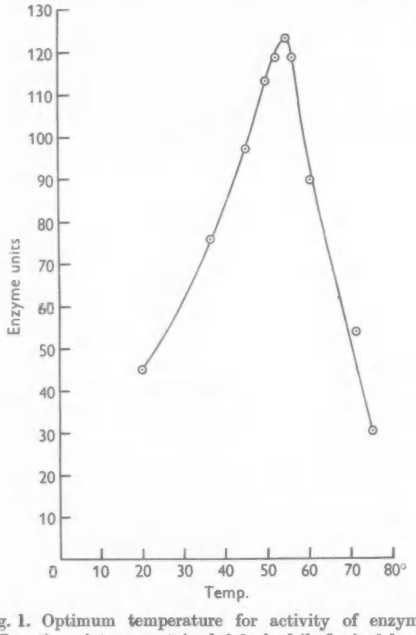

Fig. 1. Optimum temperature for activity of enzyme. Reaction mixtures contained: 0·2 ml. of riboflavin, 0·1 mM; 0·1 ml. of ATP, mM; 0·1 ml. of NaF, 0·1 M; 0·1 ml. of magnesium sulphate, 0·3 mM; 0·7 ml. of 0·1 M-veronal–HCl buffer (pH 8·6); 0·8 ml. of enzyme; total volume 2 ml. Incubated for 1 hr. at various temperatures.

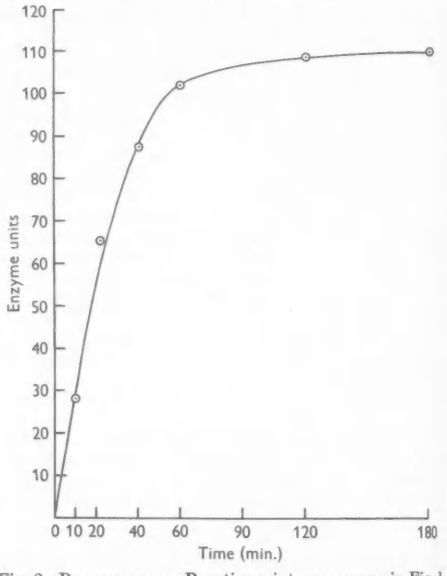

Fig. 2. Progress curve. Reaction mixtures were as in Fig. 1 and were incubated for 1 hr. at 55° for the times indicated.

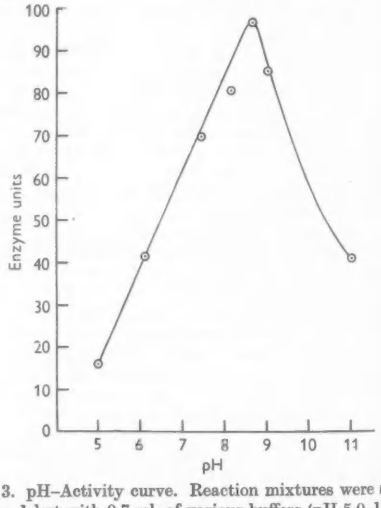

Fig. 3. pH–Activity curve. Reaction mixtures were as in Fig. 1 but with 0·7 ml. of various buffers (pH 5·0–11·0); total volume 2 ml. They were incubated for 1 hr. at 55°.

under conditions already specified, in different buffers of known pH [0·1 M-potassium hydrogen phthalate–NaOH (pH 4·0 and 5·0), 0·1 M-veronal–HCl (pH 6·0–9·0), 0·1 M-boric acid–NaOH (pH 10·0) and 0·1 M-potassium dihydrogen phosphate–NaOH (pH 11·0)].

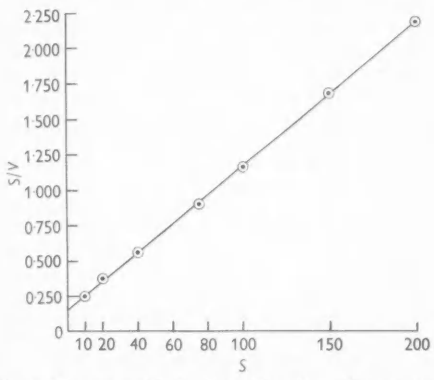

Fig. 4. Lineweaver–Burk plots. Reaction mixtures contained 0·2 ml. of riboflavin, of concentrations indicated in the graph; 0·1 ml. of ATP, mM; 0·1 ml. of NaF, 0·1M; 0·1 ml. of magnesium sulphate, 0·3 mM; 0·7 ml. of veronal–HCl buffer (pH 8·6); and 0·8 ml. of enzyme; total volume 2 ml. The mixtures were incubated for 1 hr. at 55°. *S*, Concentration of riboflavin in reaction mixtures in μmoles; *V*, μm-moles of FMN synthesized/2 ml./hr. at 55° and pH 8·6.

Table 2. *Effect of various ions on flavokinase activity*

Reaction mixtures were as follows: 0·2 ml. of riboflavin, 0·1 mM; 0·1 ml. of ATP, mM; 0·1 ml. of NaF, 0·1 M; 0·7 ml. of 0·1 M-veronal–HCl buffer (pH 8·6); 0·8 ml. of enzyme preparation from stage III and the various ions as shown in Table 2. Total volume 2 ml. Incubated at 55° for 1 hr. +, Activation; −, inhibition.

	Percentage inhibition or activation of flavokinase activity at different concentrations of ions added		
	0·1 mM	mM	10 mM
Calcium chloride	−5·1	+5·2	+5·2
Magnesium sulphate	+36·8	+26·2	+28·9
Zinc sulphate	+15·8	−2·5	−75·5
Potassium cyanide	−74·9	−74·9	−87·5
Lithium chloride	+10·5	+3·5	+0·7
Manganese sulphate	+31·5	+5·3	−26·4
Ferrous sulphate	+5·2	−31·6	−52·7
Potassium permanganate	0	−47·6	−100·0
Copper sulphate	0	+5·2	−47·6
Nickel chloride	+10·0	+5·2	+5·2
Cadmium sulphate	0	+5·2	−26·2
Cobalt acetate	0	−21·0	−42·1
Mercuric chloride	−51·9	−49·3	−63·2
	0·01 mM	0·1 mM	mM.

Various buffers such as veronal–HCl, borate–boric acid, tris, phosphate and glycine were tried at pH 8·5. Maximum amount of FMN was synthesized with veronal–HCl buffer and this buffer has been used for enzyme assay.

Substrate affinity of enzyme. Lineweaver–Burk plots are shown in Fig. 4. At a constant ATP concentration, mM, different concentrations of riboflavin were used in the reaction system.

The enzyme functions at the maximum rate at a riboflavin concentration of 0·1 mM. It was half saturated with riboflavin at a concentration of 0·015 mM. At a constant riboflavin concentration of 0·1 mM, varying concentrations of ATP were used and it was observed that the enzyme functions at a maximum rate at an ATP concentration of 2 mM.

Effect of various ions. A number of ions were tested for their effect on flavokinase activity. They were added to the system in different molar concentrations. In these experiments an enzyme preparation from stage III was used. The results are summarized in Table 2.

Of the ions used, Mg^{2+} activated the enzyme at all concentrations tested, Mn^{2+} and Zn^{2+} ions activated at low concentrations, but inhibited at higher concentrations. For routine assay of the enzyme Mg^{2+} ions were used as the activator. Kearney & Englard (1951) used Mg^{2+} ions as the standard activator for yeast flavokinase since they observed that it was the most consistent in activating the enzyme, Zn^{2+} ions having a slight effect at some stages of purification and a much greater effect at other stages of purification. As suggested by Englard (1953), Zn^{2+} ions may affect the level of ATP in the system, thus resulting in a lowered synthesis of FMN.

CN^-, Hg^{2+}, Fe^{2+}, MnO_4^- and Cu^{2+} ions were found to be inhibitory, whereas Ca^{2+}, Ni^{2+}, Li^+ and Cd^{2+} ions were without effect. Co^{2+} ions were inhibitory at concentrations higher than mM. Hydroxylamine was also found to be an inhibitor.

Distribution of flavokinase activity in plants. Examination of a number of plants indicated that flavokinase activity was present in all of those plant materials examined (Table 3).

Germination and flavokinase activity. Riboflavin is one of the vitamins which not only occurs in fairly large amounts in plants but also increases considerably during germination (Bonner, 1942). Flavokinase activity during germination of *Phaseolus radiatus* was followed and riboflavin concentrations were also determined at the various stages (Fig. 5). Seeds were allowed to germinate in the dark, in Petri dishes. They were placed on moistened filter papers cut to the same size. Water was frequently sprinkled to keep the filter paper moist. At the end of definite intervals, they were taken

out, homogenized in water (1:3, w/v), centrifuged and the supernatant obtained after dialysis of the crude extract was used as the enzyme. Riboflavin concentrations were determined on a parallel set of samples (Snell & Strong, 1939). Increase in activity of the enzyme paralleled an increase in concentration of riboflavin.

Table 3. *Occurrence of flavokinase activity in different plants*

Reaction mixtures were as follows: 0·2 ml. of riboflavin, 0·1 mM; 0·1 ml. of ATP, mM; 0·1 ml. of NaF, 0·1 M; 0·1 ml. of $MgSO_4$, 0·3 mM; 0·7 ml. of 0·1 M-veronal–HCl buffer (pH 8·4); and 0·8 ml. of enzyme from stage II. Total volume 2 ml. Incubated at 37° for 5 hr.

Plant source*	Activity†
1. *Cicer arietinum* (Bengal gram)	99·7
2. *Ricinus communis* (castor seed)	85·9
3. *Brassica campesteris* (mustard)	82·8
4. *Phaseolus mungo* (black gram)	76·7
5. *Phaseolus radiatus* (green gram)	79·7
6. *Dolichos lablab* (field bean)	148·8
7. *Dolichos biflorus* (horse gram)	99·7
8. *Canavalia gladiata* (sword bean)	118·1
9. *Pisum sativum* (peas)	99·7
10. *Sesamum indicum* (sesame)	99·7
11. *Cajanus indicus* (red gram)	99·7
12. *Solanum tuberosum* (potato)	73·6
13. *Ipomea batatas* (sweet potato)	70·0

* 1–11 were resting seeds.
† FMN synthesized at 37° and pH 8·4 (μm-moles/2 ml. of reaction mixture/5 hr.).

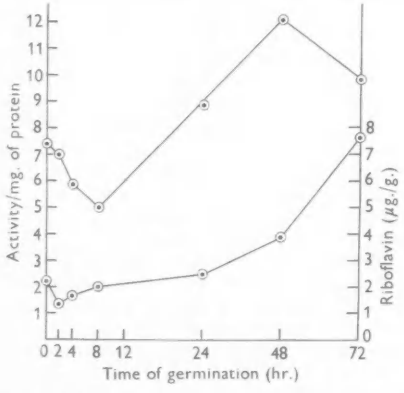

Fig. 5. Effect of germination on flavokinase activity. Flavokinase activity (top curve) and riboflavin content of green gram (*Phaseolus radiatus*) (bottom curve). Reaction mixtures were as in Fig. 1, and contained 0·8 ml. of enzyme in a total volume of 2 ml. and were incubated for 1 hr. at 55°.

DISCUSSION

The widespread occurrence of flavokinase activity in plants, as in yeasts, suggests that the main features of riboflavin metabolism are the same in plants as in yeast. Starting with riboflavin, flavin mononucleotide is synthesized by flavokinase by means of a reaction with adenosine triphosphate. The synthesis of flavin-adenine dinucleotide from riboflavin proceeds through the initial phosphorylation of riboflavin and subsequent interaction of flavin mononucleotide and adenosine triphosphate to form flavin-adenine dinucleotide by another enzymic step (Schrecker & Kornberg, 1950). During the course of these investigations synthesis of flavin-adenine dinucleotide could be demonstrated in plant extracts. Though the occurrence of riboflavin in plants is well known (Bonner, 1942; Bonner & Bonner, 1948; Watson & Nogelle, 1947), information about the enzymes concerned in its metabolism is scanty. Riboflavin is a component of many flavoprotein enzymes present in plants. Hence a study of these two enzymes which synthesize the biologically active coenzyme forms of riboflavin from plant sources may help to delineate their role in the physiology of plants.

SUMMARY

1. Flavokinase activity has been detected for the first time in plants, and its properties were studied by the application of circular-paper chromatography. The plant investigated was *Phaseolus radiatus*.

2. Optimum pH for flavokinase activity was found to be 8·6 and temperature 55°; conversion into flavin mononucleotide was nearly 60%. The Michaelis constant for riboflavin was found to be 0·015 mM.

3. Mg^{2+}, Zn^{2+} and Mn^{2+} ions showed an activating effect on flavokinase; Zn^{2+} and Mn^{2+} ions, however, were inhibitory at higher concentrations. Ca^{2+}, Ni^{2+}, Li^{+} and Cd^{2+} ions were without effect. CN^{-}, Hg^{2+}, Fe^{2+}, Cu^{2+}, MnO_4^{-} ions and hydroxylamine were inhibitory.

4. A 75-fold purification was obtained by fractionation with ammonium sulphate and adsorption with alumina-C_γ. There was a parallel increase in riboflavin levels and flavokinase activity in germinating seedlings of *Phaseolus radiatus*.

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Initial Stages in the Biosynthesis of Porphyrins

2. THE FORMATION OF δ-AMINOLAEVULIC ACID FROM GLYCINE AND SUCCINYL-COENZYME A BY PARTICLES FROM CHICKEN ERYTHROCYTES*

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It has been known for some time that the carbon skeleton of porphyrins can be synthesized *in vivo* entirely from glycine and a C_4 compound which is an intermediate in the oxidative decarboxylation of α-oxoglutarate to succinate (for review see Shemin, 1955). The discovery of the role of δ-aminolaevulic acid in porphyrin synthesis (Shemin & Russell, 1953; Neuberger & Scott, 1953; Dresel & Falk, 1953) led to the hypothesis that the initial reaction in porphyrin synthesis is the condensation of glycine with this C_4 compound to form δ-aminolaevulic acid, which is then further metabolized to porphobilinogen and porphyrins. The correctness of this view was demonstrated in Part 1 (Laver, Neuberger & Udenfriend, 1958), which described the isolation and properties of a particulate system from erythrocytes of anaemic chickens that catalyses a net synthesis of δ-aminolaevulic acid from glycine and α-oxoglutarate or succinate.

However, the nature of the reacting C_4 compound could not be determined in any of these investigations. Shemin & Wittenberg (1951) suggested that it might be succinyl-coenzyme A (succinyl-CoA) even before the isolation of this substance by Sanadi & Littlefield (1953); but little supporting evidence was obtained for this idea. Strong, but indirect evidence that coenzyme A is involved in the synthesis of δ-aminolaevulic acid comes from the study of haem and porphyrin formation in pantothenate-deficient ducks (Schulman & Richert, 1957) and *Tetrahymena vorax* (Lascelles, 1957). Coenzyme A produces also a stimulation of activity in the system studied by Laver *et al.* (1958); but all attempts to determine its role in this reaction were unsuccessful, and indeed no δ-aminolaevulic acid was formed when synthetic succinyl-coenzyme A was substituted for succinate or α-oxoglutarate.

It has now been found that, if particles prepared according to Laver *et al.* are freeze-dried, they lose most of their capacity to form δ-aminolaevulic acid from glycine and α-oxoglutarate or succinate. However, when synthetic or enzymically generated succinyl-coenzyme A is used as source of the C_4 compound, the formation of δ-aminolaevulic acid is largely restored and it appears that succinyl-coenzyme A is indeed the reactive derivative of the C_4 compound. Evidence has also been obtained which strongly implicates pyridoxal phosphate as a cofactor in this reaction. A preliminary report has appeared earlier (Gibson, Laver & Neuberger, 1958).

EXPERIMENTAL

Materials

Succinyl-coenzyme A (succinyl-CoA) was synthesized according to Simon & Shemin (1953); the product was kept at 0° until required, and was always used within a few hours. Values quoted for succinyl-CoA concentrations are nominal only and represent the amount of coenzyme A (CoA) used. Acetyl-CoA, propionyl-CoA and glutaryl-CoA were prepared similarly from the corresponding anhydrides and CoA. Crystalline glutamic dehydrogenase [10 mg./ml., suspended in 40% saturated $(NH_4)_2SO_4$] was obtained from Boehringer und Soehne, Mannheim. α-Oxoglutarate dehydrogenase was prepared according to Sanadi, Littlefield & Bock (1952); the preparation used in this work was capable of oxidizing 0·21 μmole of α-oxoglutarate/min./mg. of protein, as measured by reduction of diphosphopyridine nucleotide (DPN) in the presence of mM-cysteine and excess of succinyl-CoA deacylase (Gergely, Hele & Ramakrishnan, 1952). Crude succinic thiokinase was obtained from *Escherichia coli* (National Collection of Industrial

* Part 1: Laver, Neuberger & Udenfriend (1958).

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Bacteria, No. 8545). A suspension of this organism, grown according to Korkes (1955), except that 0·5% glucose replaced Cerelose in the growth medium, was subjected to ultrasonic disintegration on a Mullard ultrasonic generator, type E7590A (25 kc./sec. for 40 min.); the resulting suspension was clarified by centrifuging to give a pale-yellow solution which formed 3·5 μmoles of succinohydroxamic acid/hr./mg. of protein in the test system of Kaufman (1955). Phosphate buffers were made by mixing solutions of K_2HPO_4 and KH_2PO_4 as required; triethanolamine buffers were made from triethanolamine hydrochloride (obtained from Boehringer und Soehne) and NaOH. Other buffers were prepared according to Gomori (1955). Liver-coenzyme concentrate was obtained from the Armour Laboratories, Eastbourne. Aminoacetone was prepared by a modification of the stannous chloride reduction (W. G. Laver, A. Neuberger & J. J. Scott, unpublished work) of oximinoacetone; it was recrystallized from dry ethanol–ether. D-Cysteine was prepared by reduction with tin and HCl of a sample of D-cystine kindly supplied by Dr H. R. V. Arnstein of the National Institute for Medical Research, Mill Hill, London, N.W. 7. For the sources of other materials see Part I (Laver *et al.* 1958).

Methods

Preparation of freeze-dried particles. The isolation of active particles from erythrocytes of anaemic chickens was described previously (Laver *et al.* 1958). Particles prepared in this manner were freeze-dried by a conventional method and stored at −20°; under these conditions they appear to maintain their activity for at least a month. In a typical preparation, 446 mg. of freeze-dried material (including 115 mg. of KCl) was obtained from 10 ml. of packed particles. Before use, they were suspended in ice-cold water (approx. 3 ml./100 mg.) and subjected to homogenization for about 1 min. at 0° in a Potter–Elvehjem homogenizer. Amounts of freeze-dried particles used are expressed in terms of dry wt. which includes some KCl.

Incubation. Samples having a final volume of 1·7 ml. were incubated at 37° in unstoppered tubes, or *in vacuo* in Thunberg tubes. After incubation, 0·06M-iodoacetamide (0·1 ml.) was added, followed after about 1 min. by 20% trichloroacetic acid (0·6 ml.). The precipitate was removed by centrifuging, and the δ-aminolaevulic acid (ALA) content of the supernatant was estimated as described below. Addition of iodoacetamide stabilizes the colour formed with Ehrlich's reagent, probably by removing interfering thiol compounds.

Estimation of δ-aminolaevulic acid. ALA was estimated colorimetrically after condensation with acetylacetone, according to the following procedure, which was modified slightly from that of Mauzerall & Granick (1956). The 5% trichloroacetic acid filtrate (1·0 ml.) containing up to 0·1 μmole of ALA was mixed with M-sodium acetate buffer, pH 4·6 (1·0 ml.), 2·5N-NaOH (0·1 ml.), and acetylacetone (0·05 ml.). The mixture was heated at 100° for 10 min. and cooled to room temperature, and the colour was developed by addition of 2·0 ml. of the modified Ehrlich's reagent of Mauzerall & Granick (1956). After 15 min. the absorption was read at 553 mμ. Internal standards were used in each experiment. Aminoacetone was assayed by the same method.

RESULTS

Synthesis of δ-aminolaevulic acid by freeze-dried particles

When the freeze-dried particles were incubated with glycine and α-oxoglutarate and the cofactors, i.e. pyridoxal phosphate, CoA and magnesium chloride, previously found to activate ALA synthesis (Laver *et al.* 1958), there was negligible synthesis of ALA. The activity of the freeze-dried particles could be restored to a slight extent by the addition of DPN and to a greater extent by a mixture of DPN and ammonium chloride. When α-oxoglutarate was replaced by synthetic succinyl-CoA, the ability of the freeze-dried particles to synthesize ALA was greatly increased (Table 1), the amount of ALA formed probably being limited by the stability of the added succinyl-CoA at the pH and temperature of incubation. When whole particles were incubated with glycine and α-oxoglutarate under the standard conditions (Laver *et al.* 1958, Table 5), 0·240 μmole of ALA was formed by 36 mg. dry wt. of particles. When particles from the same preparation were freeze-dried, and succinyl-CoA (1 μmole) substituted for α-oxoglutarate under the conditions given in Fig. 1. 36 mg. of particles produced 0·173 μmole of ALA. Particles prepared from erythrocytes of normal, non-anaemic chickens, when freeze-dried and incubated with succinyl-CoA, glycine and pyridoxal phosphate, formed a negligible amount of ALA.

Requirements of the freeze-dried particles for synthesis of δ-aminolaevulic acid

Freeze-dried particles required the addition of both glycine and succinyl-CoA for the synthesis of ALA. There was no ALA synthesis if either of these two substrates was omitted. Fig. 1 shows the effect on ALA formation of adding increasing amounts of succinyl-CoA. It seems likely that at

Table 1. *Formation of δ-aminolaevulic acid by freeze-dried particles*

Each tube contained freeze-dried particles (30 mg.), glycine (0·06M), pyridoxal phosphate (0·2 μmole), $MgCl_2$ (3 mM), CoA (0·045 μmole), phosphate, pH 7·4 (75 mM) and other additions as described below. The final volume was 1·7 ml.; tubes were incubated for 60 min. at 37° and ALA was estimated as described.

Additions	ALA formed (μmole)
α-Oxoglutarate (0·03M)	0·004
α-Oxoglutarate (0·03M) + DPN (0·1 μmole)	0·015
α-Oxoglutarate (0·03M) + NH_4Cl (7 mM) + DPN (0·1 μmole)	0·022
α-Oxoglutarate (0·03M) + NH_4Cl (7 mM) + DPN (0·3 μmole)	0·031
Succinyl-CoA (0·3 μmole)	0·111

still higher concentrations of succinyl-CoA more ALA would have been formed, and it is probable that only the enzymes concerned in the formation of succinyl-CoA were affected by freeze-drying, while the enzyme(s) which condenses glycine with succinyl-CoA is stable. The effect of varying glycine concentration on formation of ALA (Fig. 2) is similar to that described with whole particles (Laver *et al.* 1958).

Unlike whole particles, freeze-dried particles did not require oxygen for ALA synthesis from succinyl-CoA and glycine, and no activation was obtained

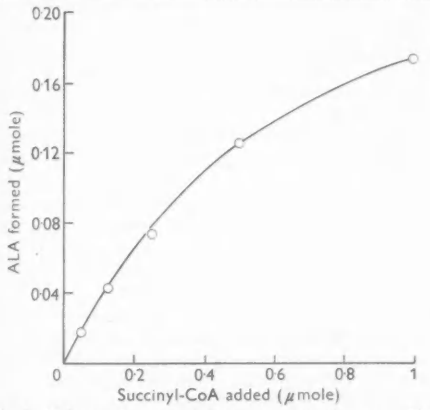

Fig. 1. Effect of succinyl-CoA concentration on ALA formation. Freeze-dried particles (32 mg.) were incubated for 60 min. at 37° with glycine (0·06 M), pyridoxal phosphate (0·2 μmole), phosphate, pH 7·4 (75 mM), and succinyl-CoA as indicated in a final volume of 1·7 ml. ALA was estimated as described.

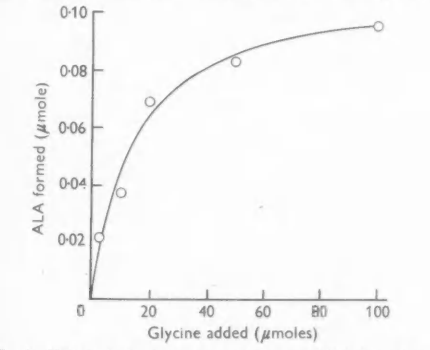

Fig. 2. Effect of glycine concentration on ALA formation. Freeze-dried particles (28 mg.) were incubated for 60 min. at 37° with succinyl-CoA (approx. 0·3 μmole), pyridoxal phosphate (0·2 μmole), phosphate, pH 7·4 (75 mM), and glycine as indicated in a final volume of 1·7 ml. ALA was estimated as described.

Table 2. *Effect of possible cofactors on the synthesis of δ-aminolaevulic acid*

The control tubes contained freeze-dried particles (approx. 30 mg.), glycine (0·06 M), pyridoxal phosphate (0·2 μmole), $MgCl_2$ (3 mM), phosphate, pH 7·4 (75 mM), and succinyl-CoA (approx. 0·3 μmole). The final volume was 1·7 ml. Omissions from the control are shown below. Tubes were incubated for 30 min. at 37°, and ALA was estimated as described.

Omissions	ALA formed (μmoles)		Test system as % of control
	Control system	Test system	
$MgCl_2$	0·096	0·093	97
Pyridoxal phosphate	0·068	0·038	56
	0·089*	0·058	65
Air	0·096*	0·087	91

* $MgCl_2$ omitted from control.

Table 3. *Effect of various buffers on synthesis of δ-aminolaevulic acid*

Each tube contained freeze-dried particles (28·5 mg.), glycine (100 μmoles), succinyl-CoA (approx. 0·3 μmole), pyridoxal phosphate (0·2 μmole) and buffer as shown, in a volume of 1·7 ml. After incubation for 30 min. at 37°, the tubes were deproteinized and ALA was estimated as described.

Buffer, pH 7·4	ALA synthesized (μmole)
Phosphate (0·09 M)	0·099
Triethanolamine (0·09 M)	0·079
2-Amino-2-hydroxymethylpropane-1:3-diol (0·09 M)	0·079
Barbitone (0·03 M)	0·047

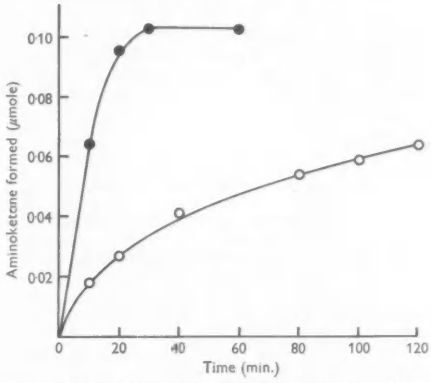

Fig. 3. Effect of time of incubation on the formation of ALA and aminoacetone. Freeze-dried particles (30 mg.) were incubated with glycine (0·06 M), pyridoxal phosphate (0·2 μmole), phosphate, pH 7·4 (75 mM), and succinyl-CoA (0·3 μmole) (●) or acetyl-CoA (0·3 μmole) (○), in a final volume of 1·7 ml. at 37° for the time indicated. ALA and aminoacetone were estimated as described.

by the addition of magnesium chloride (Table 2). The activation by phosphate observed previously with whole particles was not so marked with freeze-dried particles, but activity was still greatest when phosphate buffers were used (Table 3). The effect of time of incubation is shown in Fig. 3. The short period required for complete reaction to occur was probably the result of the instability of the added succinyl-CoA, since pre-incubation of the reaction mixture for 30 min. before the addition of succinyl-CoA did not result in any lowering of activity. The optimum pH for the reaction was found to be at about 7·4 in phosphate (Fig. 4). The requirement for pyridoxal phosphate was more marked with freeze-dried particles than previously found with whole particles (Table 2; see also Tables 6 and 8). Complete activation was obtained at a concentration of 23·5 μM of added pyridoxal phosphate, higher concentrations having no effect on the

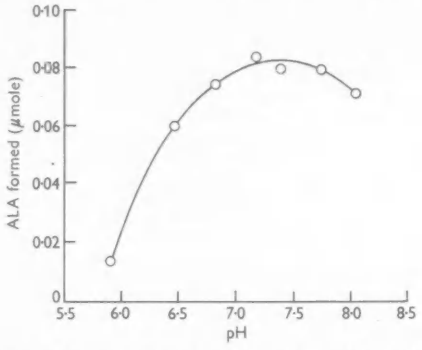

Fig. 4. Effect of pH on ALA formation. Freeze-dried particles (22 mg.) were incubated for 30 min. at 37° with glycine (0·06 M), pyridoxal phosphate (0·2 μmole), succinyl-CoA (approx. 0·2 μmole), and phosphate (0·13 M) (pH as indicated) in a final volume of 1·7 ml. ALA was estimated as described.

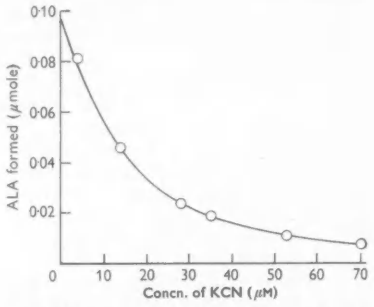

Fig. 5. Inhibition of ALA formation by cyanide. Freeze-dried particles (25 mg.) were incubated for 30 min. at 37° with glycine (0·06 M), pyridoxal phosphate (0·12 mM), succinyl-CoA (approx. 0·25 μmole), phosphate, pH 7·4 (75 mM), and KCN as indicated in a final volume of 1·7 ml. ALA was estimated as described.

Table 4. *Effect of various substances on the formation of δ-aminolaevulic acid*

Control tubes contained freeze-dried particles (approx. 30 mg.), glycine (0·06 M), pyridoxal phosphate (0·12 mM), succinyl-CoA (approx. 0·3 μmole) and phosphate, pH 7·4 (75 mM). The final volume was 1·7 ml. Additions made to the control are shown below. In the experiment with CO, the soln. was saturated with the gas which had been generated from formic acid and H_2SO_4. The tubes were incubated for 30 min. at 37° and ALA was estimated as described.

Additions	ALA formed (μmole) Test	Control	Test as % of control
p-Chloromercuribenzoate (0·44 mM)	0	0·089	0
KCN (0·18 mM)	0·004	0·084	5
KCN (0·18 mM) + pyridoxal phosphate (0·36 mM)	0·003	0·084	4
KCN (35 μM)	0·021	0·103	20
KCN (35 μM) + pyridoxal phosphate (0·36 mM)	0·021	0·103	20
L-Penicillamine (2·35 mM)	0·013	0·096	13
L-Cysteine (2·35 mM)	0·019	0·096	20
L-Cysteine (2·35 mM)*	0·021	0·115	18
D-Penicillamine (2·35 mM)	0·068	0·096	71
D-Cysteine (2·35 mM)*	0·087	0·115	76
Glutathione (2·35 mM)	0·073	0·089	82
Iodoacetamide (3·5 mM)	0·109	0·111	98
Sodium azide (1·76 mM)†	0·075	0·074	101
Sodium azide (5·28 mM)	0·073	0·074	99
$\alpha\alpha'$-Dipyridyl (0·2 mM)	0·098	0·103	95
Carbon monoxide	0·075	0·074	101
8-Hydroxyquinoline (6 mM)	0·083	0·074	112
EDTA (mM)	0·083	0·068	122

* EDTA (2·6 mM) was added to both test and control tube in these expts.
† Reaction mixture was incubated with sodium azide for 30 min. before addition of succinyl-CoA.

amount of ALA synthesized. As found with whole particles, the amount of activation varies somewhat with different preparations.

Effects of various substances on δ-aminolaevulic acid formation

The effects of various inhibitors and activators on ALA synthesis from succinyl-CoA and glycine by freeze-dried particles are shown in Table 4. Most striking was the strong inhibition by very low concentrations of cyanide, 50 % inhibition occurring at a concentration of 14 μM (Fig. 5). This inhibition could not be overcome by the addition of a large excess of pyridoxal phosphate (Table 4). Other substances which were found to inhibit the reaction were L-penicillamine and L-cysteine; D-penicillamine, D-cysteine and glutathione had a much smaller effect. Inhibition by varying concentrations of L-penicillamine is shown in Fig. 6. Sodium azide, αα′-dipyridyl and carbon monoxide had no significant effect on ALA formation, while 8-hydroxyquinoline and ethylenediaminetetra-acetic acid (EDTA) activated slightly (Table 4).

It can also be seen from Table 4 that, although iodoacetamide does not inhibit ALA formation to any significant extent, *p*-chloromercuribenzoate inhibits completely at a low concentration. It appeared possible that *p*-chloromercuribenzoate might act by splitting succinyl-CoA rather than by combining with the enzyme. This possibility was investigated in the manner described in Table 5. In the first experiment of Table 5, freeze-dried particles were incubated with *p*-chloromercuribenzoate for 5 min., after which molar quantities of succinyl-CoA varying from two-thirds to more than twice the amount of *p*-chloromercuribenzoate were added. If *p*-chloromercuribenzoate reacts stoicheiometrically with succinyl-CoA and not with the enzyme, more ALA should be formed when the amount of succinyl-CoA exceeds the amount of *p*-chloromercuribenzoate than when it does not. As can be seen, the formation of ALA was no greater in the former case than it was in the latter. In the second experiment of Table 5, succinyl-CoA was incubated with an equal amount of *p*-chloromercuribenzoate for 1 min., after which excess of glutathione was added, followed by particles. If *p*-chloromercuribenzoate acts only by splitting succinyl-CoA, there should have been a large decrease in ALA formation as compared with a control which had no *p*-chloromercuribenzoate; however, it is clear that the degree of inhibition was very small. It is concluded that, if *p*-chloromercuribenzoate does

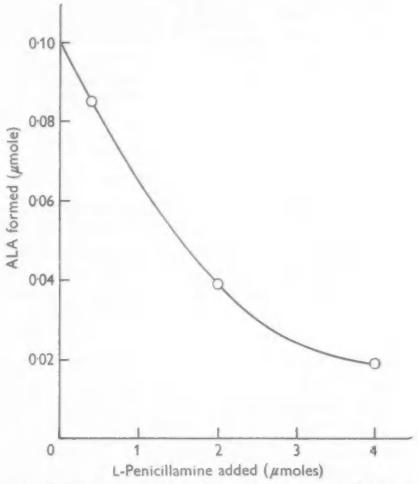

Fig. 6. Inhibition of ALA formation by L-penicillamine. Freeze-dried particles (29 mg.) were incubated for 30 min. at 37° with glycine (0·06 M), pyridoxal phosphate (0·04 μmole), succinyl-CoA (approx. 0·3 μmole), phosphate, pH 7·4 (75 mM), and L-penicillamine as indicated in a final volume of 1·7 ml. ALA was estimated as described.

Table 5. *Inhibition of the synthesis of δ-aminolaevulic acid by* p-*chloromercuribenzoate*

Each tube contained phosphate, pH 7·4 (0·09 M), glycine (100 μmoles), succinyl-CoA as indicated, freeze-dried particles (25·2 mg. in 0·6 ml.) and pyridoxal phosphate (0·2 μmole); *p*-chloromercuribenzoate and glutathione were added as shown below. The final volume was 1·7 ml. In Expt. no. 1 the reaction mixture, complete except for succinyl-CoA, was incubated at 37° for 5 min., after which the reaction was started by addition of succinyl-CoA. In Expt. no. 2, all the additions except particles and glutathione were mixed and incubated at 37° for 1 min.; at the end of this time the glutathione was added, and after a further minute the reaction was started by addition of the particles. In both experiments the reaction was stopped after 10 min., and ALA was estimated as described.

Expt. no.	*p*-Chloromercuribenzoate (μmole)	Glutathione (μmole)	Succinyl-CoA (μmole)	ALA formed (μmole)
1	0·3	—	0·2	0·016
1	0·3	—	0·4	0·018
1	0·3	—	0·8	0·011
2	0·3	0·8	0·4	0·047
2	—	0·8	0·4	0·052

Table 6. *Effect of dialysis*

In Expt. no. 1 freeze-dried particles (296 mg.) were suspended in 10 ml. of 1% KCl containing EDTA (mM) and phosphate buffer, pH 7·4 (0·01 M). A portion of this suspension (5 ml.) was dialysed against this solution (1·5 l.) for 20 hr. at 0°. Pyridoxal phosphate (0·12 mM) was added to another portion of the suspension (1·5 ml.) which was then dialysed against the same solution (100 ml. for 2 hr.; followed by 1·5 l. for 18 hr.) at 0°. The remainder of the suspension was kept at 0° for the same period.

In Expt. no. 2 freeze-dried particles (110 mg.) were suspended in distilled water containing EDTA (mM). A portion of the suspension (3 ml.) was dialysed against this solution (1 l.) for 20 hr. at 0°, while the remainder was kept at 0° for the same period.

In both experiments the dialysed and undialysed particles were incubated for 30 min. at 37° with glycine (0·06 M), phosphate, pH 7·4 (75 mM), and succinyl-CoA (approx. 0·3 μmole) in a final volume of 1·7 ml. Additions are described below; ALA was estimated as described.

Expt. no.	Freeze-dried particles	Additions	ALA formed (μmole)
1	Undialysed	None	0·046
	Undialysed	Pyridoxal phosphate (0·12 mM)	0·091
	Dialysed	None	0·056
	Dialysed	Pyridoxal phosphate (0·12 mM)	0·101
	Dialysed (pyridoxal phosphate 0·12 mM added before dialyses)	None	0·102
2	Undialysed	Pyridoxal phosphate (0·24 mM)	0·089
	Dialysed	None	0·009
	Dialysed	Pyridoxal phosphate (0·24 mM)	0·020

react with succinyl-CoA, the rate of this reaction is not sufficient to account for its inhibitory action on ALA formation. *p*-Chloromercuribenzoate therefore probably acts by direct combination with the enzyme responsible for ALA synthesis.

Effect of dialysis

The effect of dialysis on the freeze-dried particles is shown in Table 6. When a suspension of freeze-dried particles was dialysed at 0° against buffered potassium chloride containing EDTA there was no loss of activity, and the amount of activation obtained by the addition of pyridoxal phosphate was about the same as that obtained with an undialysed portion kept for the same period as control. It was also found that addition of Armour's liver coenzyme concentrate did not increase the activity of the dialysed particles. These results suggest that no diffusible cofactor is involved in the reaction. Since no loss of activity occurred on dialysis, it appeared that the endogenous pyridoxal phosphate was very strongly bound. When pyridoxal phosphate was added before dialysis, the particles were slightly more active after dialysis than they were before, although no further addition of pyridoxal phosphate was made before incubation. When the particles were dialysed against distilled water, containing EDTA, instead of against buffered potassium chloride, the activity was greatly reduced. It is likely that enzyme denaturation occurred under these conditions.

δ-Aminolaevulic acid formation from enzymically-generated succinyl-coenzyme A

It can be seen from Table 7 that freeze-dried particles form a considerable amount of ALA from

Table 7. *Formation of δ-aminolaevulic acid from glycine and succinate with succinic thiokinase*

The control tube contained freeze-dried particles (30 mg.), phosphate pH 7·4 (0·04 M), $MgCl_2$ (2·7 mM), glycine (100 μmoles), potassium succinate (25 μmoles), ATP (10 μmoles), CoA (0·05 μmole), pyridoxal phosphate (0·2 μmole), and crude succinic thiokinase from *E. coli* (4·4 mg. of protein) in a volume of 1·85 ml. After incubation for 1 hr. at 37°, the tubes were deproteinized and ALA was estimated as described. Omissions are shown below.

Omissions	ALA formed (μmole)	Activity as % of control
None	0·064	(100)
Succinate	0·005	8
CoA	0·003	5
ATP	0·002	3
E. coli extract	0·006	9
Freeze-dried particles	0·000	0

glycine and succinyl-CoA when this is generated from succinate, CoA and adenosine triphosphate (ATP) by means of succinic thiokinase from *E. coli*. In order to obtain ALA by this reaction it was necessary to add all the substrates and cofactors and succinic thiokinase; omission of succinate, CoA, ATP, thiokinase or particles reduced ALA synthesis by 90% or more. A similar experiment in which succinyl-CoA was generated from α-oxoglutarate and CoA is described in Table 8. Here use was made of the reaction catalysed by purified α-oxoglutaric dehydrogenase (Sanadi & Littlefield, 1953; Kaufman, Gilvarg, Cori & Ochoa, 1953) in which α-oxoglutarate and CoA form succinyl-CoA and carbon dioxide with concomitant reduction of DPN. Glutamic dehydrogenase and ammonia were

added in order to reoxidize reduced DPN. The table shows that more than 0·1 μmole of ALA was formed in the presence of all the additions; but, when glycine or pyridoxal phosphate was omitted, ALA synthesis decreased by 70–75%, and in the absence of added α-oxoglutarate, CoA or DPN very little ALA was formed. In contrast, the omission of α-oxoglutaric dehydrogenase or glutamic dehydrogenase led to a much smaller decrease in ALA formation. The significance of these results is discussed below.

Identification of the product of condensation of succinyl-coenzyme A and glycine

The material formed by freeze-dried particles from glycine and succinyl-CoA behaved as ALA both in the assay with acetylacetone and in the method of Shuster (1956) as modified by Laver *et al.* (1958). Good agreement between the two methods was shown in an experiment in which freeze-dried particles (80 mg.) were incubated for 1 hr. with glycine (100 μmoles), pyridoxal phosphate (0·4 μmole), succinyl-CoA (1·2 μmoles), EDTA (0·25 mM) and phosphate (0·03 M), pH 7·4, in a final volume of 3·0 ml. In a control tube from which succinyl-CoA was omitted, 0·2 μmole of ALA was added to serve as an internal standard. The amount of ALA formed in the experimental tube was estimated as 0·227 μmole by the modified picric acid method and as 0·174 μmole when assayed by condensation with acetylacetone. None of the pyrrole formed in this latter reaction could be extracted into ether at pH 8. In this experiment iodoacetamide was not added before assay and the lower figure obtained by the second method may have been due to interference in the colour reaction by the thiol group of CoA.

Table 8. *Formation of δ-aminolaevulic acid from glycine and α-oxoglutarate with α-oxoglutaric dehydrogenase*

The control tube contained freeze-dried particles (24·0 mg.), phosphate, pH 7·4 (0·09 M), glutathione (1·5 μmole), glycine (100 μmoles), pyridoxal phosphate (0·2 μmole), CoA (0·03 μmole), potassium α-oxoglutarate (5 μmoles), DPN (0·2 μmole), α-oxoglutaric dehydrogenase (49 μg.), glutamic dehydrogenase (100 μg.), and $(NH_4)_2SO_4$ (20 μmoles) in a volume of 1·7 ml. After incubation for 1 hr. at 37°, the tubes were deproteinized and ALA was estimated as described. Omissions are indicated below.

Omissions	ALA formed (μmole)	Activity as % of control
None	0·115	(100)
Glycine	0·028	24
α-Oxoglutarate	0·012	10
CoA	0·017	15
Pyridoxal phosphate	0·034	30
DPN	0·016	14
α-Oxoglutaric dehydrogenase	0·075	65
Glutamic dehydrogenase	0·082	71

Fractionation of freeze-dried particles

Freeze-dried particles (327 mg.) were suspended in water (10 ml.) and homogenized, as described under Methods. When this extract (1 ml.) was incubated for 1·5 hr. with glycine, succinyl-CoA and pyridoxal phosphate under the same conditions as in Table 4, 0·083 μmole of ALA was formed. The supernatant obtained by centrifuging this extract (1500 *g*; 20 min.) when incubated under identical conditions formed 0·041 μmole of ALA and the supernatant obtained by centrifuging the extract at high speed (20 000 *g*; 15 min.) formed 0·038 μmole of ALA. It appeared therefore that part of the activity was soluble, but no further fractionation of the soluble material was attempted.

Formation of aminoacetone and analogous amino ketones by freeze-dried particles

Shemin (1955) suggested that the condensation of glycine with succinate to form ALA might be only one example of a more general reaction. The ability of acetyl-CoA, propionyl-CoA and glutaryl-CoA to form amino ketones with glycine was therefore investigated. When synthetic acetyl-CoA was incubated with freeze-dried particles, together with glycine and pyridoxal phosphate, a substance was formed which reacted with acetylacetone at pH 4·6 to give a pyrrole that could be extracted into ether at pH 8. It was found that there was no disappearance of synthetic aminoacetone during incubation at a concentration of 0·3 mM with particles for 1 hr. at 38° and pH 7·4, and that it could be assayed by condensation with acetylacetone at pH 4·6 by using exactly the same method as that employed for ALA. The pyrrole resulting from this condensation, having no free carboxyl group, could be completely extracted into ether at pH 8. The amino ketone formed from acetyl-CoA and glycine may therefore be presumed to be aminoacetone. The effect of time of incubation on the formation of aminoacetone is shown in Fig. 3. Freeze-dried particles form much less aminoacetone than they do ALA. The fact that enzymic synthesis continues for a longer period is probably due to the greater stability of acetyl-CoA compared with that of succinyl-CoA.

Propionyl-CoA and glutaryl-CoA were also found to give rise to amino ketones when incubated with glycine, pyridoxal phosphate and freeze-dried particles. Since authentic samples of 6-amino-5-oxohexanoic acid and 1-aminobutan-2-one were not available, the amounts of these substances formed enzymically could not be estimated quantitatively, but appreciably less colour was produced with Ehrlich's reagent than in the case of ALA.

Formation of aminoacetone from pyruvate by whole particles

Whole particles (0·6 ml.) were incubated for 1·5 hr. with pyruvate (3 mM), glycine (0·06 M), pyridoxal phosphate (0·2 μmole), CoA (0·05 μmole), $MgCl_2$ (3 mM) and phosphate, pH 7·4 (65 mM), in a final volume of 1·7 ml. Under these conditions 0·029 μmole of aminoacetone was formed. When pyruvate was replaced by 3 mM-α-oxoglutarate, 0·257 μmole of ALA was formed under identical conditions.

DISCUSSION

Activation of succinate and α-oxoglutarate

It was shown in Part 1 (Laver *et al.* 1958) that washed whole particles obtained from the lysates of erythrocytes of chickens, which had been made anaemic, can synthesize ALA from α-oxoglutarate (or succinate) and glycine. No ALA was formed on incubation of the particles with glycine and succinyl-CoA. The present paper deals with the behaviour of preparations obtained from freeze-dried particles. Such preparations are inactive on incubation with α-oxoglutarate, glycine, coenzyme A, magnesium chloride and phosphate (Table 1), but a relatively small amount of ALA is formed on addition of DPN or of DPN and ammonium chloride. When α-oxoglutarate in this system is replaced by succinyl-CoA, a further and very considerable increase in activity is found. Indeed, the amount of ALA formed under these conditions with freeze-dried preparations is only slightly smaller than that obtained with whole particles and α-oxoglutarate. Whole particles were found to be active without addition of CoA and addition of this coenzyme produced on the average an activation of 20 or 45 % only with α-oxoglutarate and succinate respectively. With freeze-dried preparations, on the other hand, there is no ALA formation unless succinyl-CoA is added as such (Fig. 1) or is enzymically produced in the system either by the action of succinic thiokinase of *E. coli* on succinate (Table 7) or by the action of purified α-oxoglutaric dehydrogenase on α-oxoglutarate (Table 8).

It must be presumed therefore that succinyl-CoA is the intermediate in ALA formation in whole particles as well as in freeze-dried preparations. The formation of succinyl-CoA in whole particles is almost certainly due to α-oxoglutaric dehydrogenase. The presence of α-oxoglutaric dehydrogenase in freeze-dried preparations can be inferred from the fact that ALA formation from α-oxoglutarate is markedly stimulated by addition of DPN (Table 1), the known hydrogen-acceptor in this reaction (Sanadi & Littlefield, 1953; Kaufman *et al.* 1953). The further increase of ALA synthesis produced by addition of ammonium ions to this system implies that freeze-dried preparations also contain glutamic dehydrogenase, which reoxidizes reduced DPN while forming glutamate from α-oxoglutarate and ammonia. The correctness of this view is borne out by the experiments reported in Table 8, in which purified preparations of these two enzymes were added. Omission of only one of these enzymes resulted in a relatively small decrease in ALA formation, indicating that the freeze-dried preparations contain both α-oxoglutaric dehydrogenase and glutamic dehydrogenase. When DPN was left out, there was a great reduction in ALA synthesis, which is to be expected if α-oxoglutaric dehydrogenase is involved.

In the earlier paper (Laver *et al.* 1958) it was reported that with whole particles DPN had a slight, but definite, activating effect in the absence of oxygen. The smallness of this effect and the non-availability of succinyl-CoA to whole particles is most satisfactorily explained by the existence of permeability barriers, which disappear on freeze-drying and on mechanical homogenization (Gibson *et al.* 1958). The fact that ALA formation from α-oxoglutarate in whole particles is not inhibited by azide may also be explained in terms of a permeability barrier. Alternatively, the oxidation of reduced DPN may occur through enzymes other than those of the cytochrome system, such as the oxidases which have been found in *Streptococcus faecalis* (Dolin, 1955).

The path by which succinate is used for ALA synthesis in whole particles is not clearly established. The work reported in Part 1 and the results shown in Table 7 are consistent with the data of Shemin & Kumin (1952), which indicate that the conversion of succinate into succinyl-CoA in normal duck red cells occurs mainly by oxidation through the Krebs cycle. Their figures show that there is in addition a direct conversion of succinate into its CoA derivative. Although the latter reaction may contribute to ALA formation from succinate in whole particles, the results shown in Table 7 indicate that there is little or no thiokinase in freeze-dried preparations.

Role of pyridoxal phosphate

The evidence that pyridoxal phosphate is a cofactor in the synthesis of ALA was briefly discussed in Part 1. As with whole particles, the decrease in ALA formation which occurred when pyridoxal phosphate was not added to the test system varied from one freeze-dried preparation to another; but there was always a decrease of at least 30 % and usually of 40–50 % (see Table 2). In one case the decrease was 70 % (Table 8). Similar results were reported for ALA formation by homogenized preparations (Gibson *et al.* 1958). The results reported in Table 6 show that added pyrid-

oxal phosphate is bound in such a way that it is not removed by dialysis and that it is the bound pyridoxal phosphate that acts catalytically in ALA formation. The finding that freeze-dried preparations are active without addition of pyridoxal phosphate, and that this activity is not decreased by dialysis against buffered potassium chloride solution, indicates that these preparations contain bound pyridoxal phosphate.

The conclusion that enzyme-bound pyridoxal phosphate is a cofactor in ALA synthesis will also explain results obtained with penicillamine, cysteine and cyanide. There is no evidence from the present work that the inhibitory effect of penicillamine is due to its combination with pyridoxal phosphate; but this is likely on chemical grounds, as du Vigneaud, Kuchinskas & Horvath (1957) have shown that these compounds rapidly form a thiazolidine compound under physiological conditions. These authors have also shown that liver transaminases, which contain pyridoxal phosphate as prosthetic group, are strongly inhibited by L-penicillamine. The enzyme system which forms ALA from succinyl-CoA and glycine has approximately the same sensitivity to L-penicillamine as have the transaminases studied by du Vigneaud *et al.* (1957). D-Penicillamine is a much less effective inhibitor. The steric specificity of this inhibition is consistent with the conclusion drawn above that the catalytically active pryridoxal phosphate is bound to protein. The effects shown by L- and D-cysteine closely resemble those shown by the two isomers of penicillamine. The fact that glutathione inhibits only slightly accords with the suggestion of du Vigneaud *et al.* (1957) that the effect of penicillamine is caused by thiazolidine formation with pyridoxal phosphate.

The formation of ALA from succinyl-CoA and glycine by freeze-dried preparations is inhibited by cyanide to an even greater extent than the synthesis by whole particles from α-oxoglutarate and glycine. It follows that cyanide inhibits the condensation reaction. Furthermore, this inhibition is unlikely to be due to some reaction with succinyl-CoA, since an inhibition of 80% was observed under conditions in which the molar concentration of succinyl-CoA was about five times that of cyanide (Table 4). It is therefore concluded that cyanide inhibits by reaction with pyridoxal phosphate. Enzymes which contain pyridoxal phosphate are inhibited to a greater or lesser degree by cyanide. Of all the bacterial amino acid decarboxylases studied by Taylor & Gale (1945) only glutamic and lysine decarboxylases approach the ALA-forming enzyme in sensitivity to cyanide. Diaminopimelic decarboxylase, which almost certainly contains pyridoxal phosphate (Dewey, Hoare & Work, 1954), is considerably less sensitive to cyanide, as are liver transaminases (Cohen, 1939; Vyshepan, 1940) and kynureninase (Jakoby & Bonner, 1953). The finding that inhibition by cyanide is not reversed by an excess of pyridoxal phosphate is consistent with the assumption that cyanide has a much greater affinity for enzyme-bound than for free pyridoxal phosphate. It is generally assumed (see, for example, Gale, 1946) that cyanide inhibits pyridoxal phosphate enzymes through cyanohydrin formation, but the facts just discussed suggest that the active centre of the enzyme contains a metal in addition to pyridoxal phosphate (Metzler, Ikawa & Snell, 1954) and that cyanide reacts with this complex. However, it should be pointed out that no inhibition, but a slight activation, was observed on addition of EDTA or 8-hydroxyquinoline and no effect was obtained with azide and αα′-dipyridyl (Table 4).

Mechanism of the reaction

No direct evidence has been obtained in the present work concerning the activation of the α-carbon atom of glycine, and the possibility that glycyl-CoA, formed by transfer from succinyl-CoA, is the reactive intermediate, cannot at this stage be ruled out. However, the simplest explanation, consistent with the evidence presented, is that the reactive form of glycine is a Schiff's base formed between this amino acid and pyridoxal phosphate bound to the enzyme and that this complex reacts with succinyl-CoA. A considerable amount of work has been done on the Schiff's bases formed under physiological conditions between pyridoxal or pyridoxal phosphate and various amino acids, including glycine (e.g. Blakley, 1955; Matsuo, 1957; Christensen, 1958). The classical studies of Erlenmeyer & Früstück (1895) and of Erlenmeyer (1899) showed that formation of a C–C bond involving the α-carbon atom of glycine is greatly facilitated by prior condensation between the amino group and an aldehyde. More recently it has been shown by Metzler, Longenecker & Snell (1953, 1954) that pyridoxal and a metal catalyse the non-enzymic condensation of formaldehyde and glycine. The reversible enzymic transformation of glycine into serine is also dependent on the presence of pyridoxal phosphate (Blakley, 1954, 1955; Heunnekens, Hatefi & Kay, 1957; see also Kisliuk & Sakami, 1955) and so is the enzymic conversion of glycine and acetaldehyde into threonine (Braunshtein & Vilenkina, 1953) and allothreonine (Karasek & Greenberg, 1957). It is now postulated that pyridoxal phosphate fulfils a similar function in the acyl addition involved in ALA synthesis as it does in the aldol condensations discussed above.

The primary product of the condensation has not been identified. It has been suggested (Shemin & Russell, 1953; Neuberger & Scott, 1953) that

α-amino-β-oxoadipate is an intermediate in ALA formation, but the ease with which this compound loses carbon dioxide spontaneously after chemical synthesis has so far prevented its isolation (W. G. Laver, A. Neuberger & J. J. Scott, unpublished work). There is therefore no information as to whether it is enzymically decarboxylated. Even if it should be possible to show such enzymic action, it could not be concluded that α-amino-β-oxoadipate is an intermediate. Although highly purified *iso*citric dehydrogenase catalyses the decarboxylation of oxalosuccinate (Moyle & Dixon, 1956; Siebert, Carsiotis & Plaut, 1957) it has been shown by means of isotopes that this compound is not an intermediate in the overall oxidation of *iso*citrate to oxoglutarate (Siebert *et al.* 1957). Similarly, oxaloacetate is not intermediate in the synthesis of malate from pyruvate and carbon dioxide by the 'malate' enzyme (Veiga Salles, Harary, Banfi & Ochoa, 1950; Harary, Korey & Ochoa, 1953).

Freeze-dried preparations have been found to form substances reacting as amino ketones when succinyl-CoA is replaced by other acyl-CoA derivatives. The reaction with acetyl-CoA is much slower than with succinyl-CoA (Fig. 3) and it is probable that propionyl-CoA and glutaryl-CoA react even more sluggishly. It is not known whether all these reactions are catalysed by the same enzyme. It is possible that the reaction with acetyl-CoA has some physiological significance, as suggested by Nemeth, Russell & Shemin (1957), since some aminoacetone is formed on incubation of whole particles with glycine, pyruvate, CoA and pyridoxal phosphate.

Inhibition with thiol reagents

The synthesis of ALA from oxoglutarate and glycine by whole particles was markedly inhibited by iodoacetamide and *p*-chloromercuribenzoate (Laver *et al.* 1958). This is probably due to interaction with coenzyme A. With freeze-dried particles and succinyl-CoA, iodoacetamide is without effect, but *p*-chloromercuribenzoate still inhibits (Table 4), almost certainly by direct combination with the enzyme (Table 5). This inhibition may not be caused by interaction with thiol groups, as it has been shown that inhibition of catalase by organic mercurials is due to causes other than blocking of thiol groups (Sohler, Seibert, Kreke & Cook, 1952). On the other hand, betaine aldehyde dehydrogenase, which is known to be a thiol enzyme, is strongly inhibited by *p*-chloromercuribenzoate, but is much less sensitive to iodoacetamide and iodoacetate (Rothschild & Barron, 1954). The question whether the enzyme responsible for the condensation of succinyl-CoA and glycine has an essential thiol group must therefore be left open.

SUMMARY

1. Preparations of freeze-dried particles obtained from erythrocytes of anaemic chickens synthesize δ-aminolaevulic acid from succinyl-coenzyme A and glycine. This is true, whether succinyl-coenzyme A is chemically synthesized or generated enzymically from succinate or α-oxoglutarate.

2. Such preparations form little or no δ-aminolaevulic acid from α-oxoglutarate, unless diphosphopyridine nucleotide is added. It is concluded from this and other evidence that the synthesis of δ-aminolaevulic acid from α-oxoglutarate in whole particles, described by Laver *et al.* (1958), involves the formation of succinyl-coenzyme A by the action of α-oxoglutaric dehydrogenase.

3. The addition of pyridoxal phosphate stimulates the synthesis of δ-aminolaevulic acid 1·5-3-fold. Evidence was obtained that pyridoxal phosphate is firmly bound to the enzyme. No other cofactor appears to be involved in the condensation of succinyl-coenzyme A and glycine.

4. *p*-Chloromercuribenzoate, cyanide, L-penicillamine and L-cysteine inhibit the reaction strongly. D-Penicillamine, D-cysteine and glutathione inhibit slightly; iodoacetamide, sodium azide, αα′-dipyridyl and carbon monoxide have no effect, while 8-hydroxyquinoline and ethylenediaminetetra-acetate activate slightly. Inhibition by cyanide is unchanged in the presence of a tenfold excess of pyridoxal phosphate.

5. Freeze-dried particles form aminoacetone on incubation with glycine, acetyl-coenzyme A and pyridoxal phosphate, and substances reacting as aminoketones when acetyl-coenzyme A is replaced by propionyl- and glutaryl-coenzyme A. Whole particles form aminoacetone from pyruvate and glycine. These reactions are slower than the synthesis of δ-aminolaevulic acid.

6. It is suggested that the α-carbon of glycine is activated, before condensing with succinyl-coenzyme A, by the formation of a Schiff's base between the amino group of glycine and enzyme-bound pyridoxal phosphate. The inhibition of the synthesis of δ-aminolaevulic acid by cyanide, penicillamine and cysteine is explained on this basis.

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Studies on the Proteins of Fish Skeletal Muscle

5. MOLECULAR WEIGHT AND SHAPE OF COD FIBRILLAR PROTEINS†

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It was shown in a previous paper in this series (Connell, 1958) that unaggregated myosin is present in extracts of cod skeletal muscle, and that attempts to isolate the monomeric protein from the extracts by employing the usual method of precipitating by dilution failed because it aggregated spontaneously. It has now been confirmed that the tendency of cod myosin to aggregate is much greater than that of the rabbit protein. Measurements of the molecular weight and shape of unaggregated cod myosin have been undertaken therefore to decide whether some feature of these parameters could explain the unusual behaviour of the fish protein.

Although many attempts to prepare unaggregated cod myosin from extracts containing it have

† Part 4: Connell (1958).

been made in this Laboratory, none has proved successful. However, it has been found possible to prepare monodisperse cod myosin by separating it in the preparative ultracentrifuge after the dissociation of actomyosin with adenosine triphosphate (ATP) or sodium pyrophosphate at 0°. The method stems from Weber's (1956) work on the ultracentrifugal separation of rabbit myosin and Hamoir's (1951) finding that treatment of carp actomyosin with ATP gives rise in the ultracentrifuge to a single component whose sedimentation rate corresponds to that of rabbit myosin.

Although it is possible to determine accurately the sedimentation coefficient of monodisperse cod myosin, the instability of the protein renders the determination of diffusion coefficients less accurate, and it was therefore important to obtain a value for the molecular weight independent of the diffusion coefficient. Archibald's (1947) procedure of measuring the pre-equilibrium conditions obtaining in an ultracentrifuged solution as developed for proteins by Klainer & Kegeles (1955) and Ginsburg, Appel & Schachman (1956) offers an ideally rapid method of molecular-weight determination, and it has been therefore applied to cod myosin.

The molecular weight of an impure preparation of cod G-actin appeared to be near that of rabbit G-actin dimer (Connell, 1954), but it was decided to reinvestigate this by purifying the cod protein according to Mommaerts' (1952) procedure and examining its sedimentation behaviour.

Natural rabbit actomyosin, that is the actomyosin prepared by extracting fresh rabbit muscle, appears to be polydisperse (Weber & Portzehl, 1952*a*) and there seems little justification for attempting to obtain an accurate value for the molecular weight of this complex. On the other hand, Hamoir (1951, 1955*a*, *b*) speaks of carp actomyosin as consisting of one component. Also, the importance which natural actomyosin has assumed as a model substance in experiments designed to study the effects of technological processes on meat and fish demands that more information about this complex be obtained. In the present investigation, the order of the molecular weight of cod actomyosin has been estimated from turbidity measurements at different wavelengths.

EXPERIMENTAL

Materials

Buffers. A buffer of pH 7·5 and ionic strength 0·05 of the following composition was used: 3·38 mM-KH_2PO_4–15·5 mM-Na_2HPO_4. Potassium chloride or iodide was added to this buffer to bring the total ionic strength up to the values quoted below. Sodium thiosulphate (6 mM) was added to the buffers containing KI, to guard against the liberation of free iodine.

Myosin. All preparative operations were conducted near 0°. A mixture of cod myosin and its aggregates was prepared from skeletal muscle excised from 'live' cod (*Gadus callarias* L.) which had been killed only a few minutes previously. The muscle was coarsely minced and extracted with 3 vol. of slowly stirred KCl–phosphate buffer ($I = 0{\cdot}3$) for 10 min. The mixture was centrifuged at 3000 *g* for 15 min. and the supernatant solution diluted by the slow addition during stirring of 6 vol. of water. The precipitate generally showed a 'sheen' on stirring. Precipitation was allowed to proceed for 1 hr. although longer periods sometimes appear to be necessary, and the precipitate was collected by centrifuging at 3000 *g* for 15 min. The pad of myosin was dissolved by the addition of an equal volume of KCl–phosphate buffer ($I = 1{\cdot}0$) and the protein was re-precipitated by the addition of 10 vol. of water as above. After dissolving the centrifuged precipitate the solution was either examined immediately, or dialysed against KCl–phosphate buffer ($I = 0{\cdot}5$).

Monodisperse myosin was prepared from cod actomyosin prepared as described below. The actomyosin was prepared either from muscle excised immediately after the death of the fish or from muscle which had been frozen *pre-rigor*. Apart from the smaller yield of actomyosin obtained from muscle frozen *pre-rigor* no difference in either the actomyosin or the myosin obtained therefrom has ever been detected between these two types of muscle. The final solution of actomyosin used for the preparation of myosin was dialysed against KCl–phosphate buffer ($I = 0{\cdot}5$) for no longer than about 20 hr., because some evidence has been obtained that small quantities of myosin which contaminate the actomyosin aggregate during this period and appear in the final myosin supernatant solution. In addition the actomyosin itself aggregates and the yield falls. To the dialysed actomyosin solution was added either 5 mM-ATP + 5 mM-$MgCl_2$ or mM-sodium pyrophosphate + mM-$MgCl_2$. The reagents were all neutralized and their volume was sufficiently small to affect the concentration of buffer only negligibly. The treated actomyosin was immediately centrifuged in a cooled preparative 'A' rotor of a Spinco ultracentrifuge at an average of 102 000 *g* for 2¾ hr. The centrifuge tubes then contained a water-clear supernatant solution, an almost transparent and gel-like pellet and a small white opaque precipitate at the tip. The top 40–50% layers of the supernatant myosin solution were immediately removed. Tubes containing untreated cod actomyosin centrifuged in the same way contained a much looser and more opaque pellet and a more opaque supernatant solution whose opacity decreased towards the meniscus.

So far twenty-nine preparations have been made in this way with substantially the same result; in most of them pyrophosphate was used because of the expense of ATP. If the concentration of actomyosin in the treated solution exceeded about 0·7% it contaminated the myosin presumably because the dissociation was incomplete. The concentration of the myosin solution was never more than about 0·4%, which has imposed restrictions on the range of observations possible with the protein since it must be examined immediately if it is to remain monodisperse.

The viscosity of myosin solutions obtained by treating cod actomyosin with ATP did not fall when they were treated with ATP (the test cannot be applied to solutions containing pyrophosphate), indicating the virtual absence of

actomyosin. Ultracentrifugal analyses of myosin solutions prepared by dissociating cod actomyosin almost always showed a single slowly diffusing component (Fig. 1*a*). Occasionally traces of much heavier or lighter components are discernible, presumably actomyosin and sarcoplasmic proteins unremoved from the original actomyosin respectively. The area (corrected for dilution in a sector-shaped cell) of the myosin peak in the ultracentrifuge picture falls by about 5–10% during a run; this may be due to some systematic error in the subtraction of the base line of the picture or to the presence in the myosin of small amounts of material which have approximately the same sedimentation rate and which give rise to very rapidly spreading boundaries. No difference between the myosins prepared from actomyosin treated with either ATP or pyrophosphate has been detected (see Results).

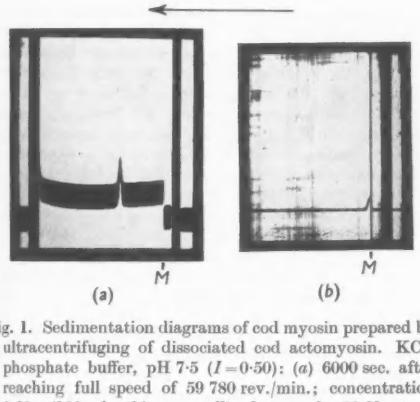

Fig. 1. Sedimentation diagrams of cod myosin prepared by ultracentrifuging of dissociated cod actomyosin. KCl–phosphate buffer, pH 7·5 ($I = 0{\cdot}50$): (*a*) 6000 sec. after reaching full speed of 59 780 rev./min.; concentration 0·28 g./100 ml. (12 mm. cell); bar angle 70·0°; temperature 1·00°. (*b*) 4320 sec. after reaching full speed of 7247 rev./min.; concentration 0·40 g./100 ml. (18 mm. cell); wire angle 70·0°; temperature 4·60°. *M* denotes the position of the meniscus.

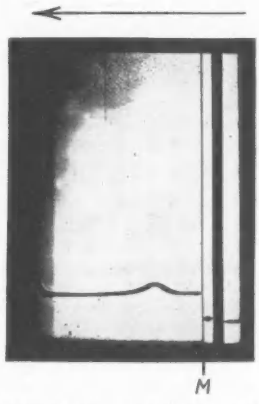

Fig. 2. Sedimentation diagram of ultracentrifugally purified cod G-actin in 0·1 mM-ATP, pH 8·5, 8220 sec. after reaching full speed of 59 780 rev./min.; concentration 0·34 g./100 ml. (12 mm. cell); wire angle 70·0°; temperature 1·15°. *M* denotes the position of the meniscus.

Attempts to apply Szent-Györgyi & Szent-Györgyi's (1956) KI method of preparing myosin to fish yielded only traces of protein resembling myosin.

Actin. Acetone-dried powders were prepared by Bárány, Bárány & Guba's (1957) procedure from muscle removed from cod killed a few minutes before. The powder was extracted for 1 hr. at room temperature with 20 vol. of water, which was stirred occasionally by hand. The paste was filtered through a Büchner funnel and the filtrate, containing 0·3–0·5% of protein, was treated substantially according to Mommaerts' (1952) procedure. This procedure involves polymerizing the actin and separating it from light unpolymerized protein by high-speed centrifuging. Two polymerizations were carried out: about 75 and 90% of the actin polymerized and could be centrifuged in the first and second polymerizations respectively. The F-actin was depolymerized in 0·1 mM-ATP adjusted to pH 8–8·5 with $NaHCO_3$. The final solution, containing 0·3–0·5% of protein, was dialysed against either 0·1 mM-ATP, pH 8–8·5, or KI–phosphate buffer ($I = 0{\cdot}66$) to continue the depolymerization, or against KCl–phosphate buffer ($I = 0{\cdot}3$ or 0·5) to repolymerize the actin. Ultracentrifugal analysis of the crude aqueous extract of powder showed the presence of two heterogeneous components having nearly the same rate of sedimentation, but the purified preparations showed only a single diffuse boundary (Fig. 2).

Actomyosin. Coarsely minced muscle either from cod killed only a few minutes beforehand or frozen *pre-rigor* was extracted for 18–24 hr. with 5–10 vol. of KCl–phosphate buffer ($I = 0{\cdot}5$), with slow mechanical stirring. The brei was centrifuged at 300 *g* for 30 min. and the cloudy, very viscous supernatant solution diluted by the addition, with stirring, of 10 vol. of water. After allowing to stand ½–1 hr. the precipitate was collected by centrifuging at 3000 *g* for 30 min. and redissolved by the addition of an equal volume of KCl–phosphate buffer ($I = 1{\cdot}0$). After one or two further precipitations in the same way, the final solution was dialysed against KCl–phosphate buffer ($I = 0{\cdot}5$). For the turbidity measurements only unfrozen muscle was used, and the final dialysis was for 2–3 days against several changes of 0·5 M-KCl.

Methods

Ultracentrifuging. The Spinco model E equipped with a Rotor-Temperature Indicator and Control unit was used. With very few exceptions (indicated below) the temperature of the runs has been kept between 0 and 5°, and the samples have been loaded into precooled rotors. For runs requiring a knowledge of the whole refractive-index gradient in the cell (e.g. Archibald, 1947 procedure), the bar of the schlieren set up was replaced by a wire of 0·0089 cm. diameter (43 s.w.g.), and measurements were taken at the mid-point of the photographed image of this wire. The photographic plates were measured with a two-way travelling microscope reading to 0·01 mm.

Sedimentation coefficients (S) were calculated as follows from photographs taken at intervals of 16 or 32 min.:

$$S = \frac{\Delta x}{\Delta t}\frac{1}{\bar{x}\omega^2} \quad \text{or} \quad \frac{ln\,(x_2/x_1)}{\Delta t\omega^2},$$

where Δx is the distance moved by the maximum ordinate of the boundary from radial distances of x_1 to x_2 in a time interval of Δt, $\bar{x}$ is the mean radial distance and ω the angular velocity. No tendency of the values of S calculated as above either to increase or decrease during a run could be detected and therefore they were averaged for a single run. The average values of S were corrected to a liquid of the density and viscosity of water at 20° (Svedberg & Pedersen, 1940). The values at 20° of the partial specific volume used in this correction were 0·730 and 0·74 for cod myosin (see below) and actin respectively. The latter value is assumed and it is also assumed that the partial specific volume of myosin falls by 0·0005 per degree (Svedberg & Pedersen, 1940, Appendix II; Noda, Kuby & Lardy, 1954).

Archibald procedure. The procedure was essentially that of Klainer & Kegeles (1955) and Ginsburg *et al.* (1956) and makes use of the relationship:

$$M = \frac{RT}{(1-\bar{v}\rho)} \frac{(\partial c/\partial x)_{x_0}}{x_0 \omega^2 c_{x_0}},$$

where M is the molecular weight of the solute, R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume of the solute, ρ is the density of the solution, $(\partial c/\partial x)_{x_0}$ is the value of the concentration gradient at either end of the centrifuged column of solution, x_0 is the radial distance of either end of the column, ω is the angular velocity and c_{x_0} is the concentration of solute at either end of the column.

In order to improve the definition of the image of the diagonal wire in these runs, the cell path-length was increased to 18 mm., which enabled larger angles of the wire (to the slit image at the wire) to be used. With this arrangement values of $(\partial n/\partial x)_{x_0}$, the refractive-index gradient at the top meniscus of the cell, of 0·2–0·6 cm. at the photographic plate were obtained for myosin of the greatest concentration available (about 0·4%). The total concentration of myosin was determined (in arbitrary units on the photographic plate) in a 12 mm. synthetic boundary cell and the values were multiplied by 1·5 in order to correct to the 18 mm. cell.

The photographic plates were aligned so that the middle of the image of the top meniscus furnished one co-ordinate and measurements of $\partial n/\partial x$ taken at 0·1 mm. intervals from this co-ordinate. At the speeds used (9230 and 7247 rev./min. in the analytical B rotor), the photographic image of the meniscus was itself about 0·1–0·2 mm. broad, so that uncertainty about its exact position might make up to 5% error in the determination of M (Cheng, 1957). Apart from the very early stages of a run (where it was not possible to make measurements) the refractive-index-gradient curve produced by myosin showed (Fig. 1*b*) at a distance of about 0·4–0·5 mm. from the meniscus a definite inflexion which is not apparent in the curve produced by low-molecular-weight proteins (e.g. bovine serum albumin) under the same conditions. Therefore the method of straight-line extrapolation used by Ginsburg *et al.* (1956) was not adopted, and instead the experimental points were plotted up to a distance of 0·2 mm. towards the position of the top meniscus. A smooth curve was drawn freehand through these points and continued up to the meniscus in order to obtain values of $\partial n/\partial c$ at the meniscus and at a point 0·1 mm. from it (Fig. 3). This procedure will introduce further errors into the determination of M, though they will probably at most be 5%.

It has been found possible to utilize only the gradient at the top meniscus in the calculation of M, since the bottom of the cell is not registered on the photographic plate and neither carbon tetrachloride nor dense silicone fluids could be used to form a registerable bottom meniscus, because they appeared to aggregate or precipitate cod myosin (but not bovine serum albumin). With the conditions described above, the average molecular weight of bovine serum albumin (The Armour Laboratories, Chicago, Ill., U.S.A.) was found to be 74 500, which is 6–7% higher than the generally accepted figure for this protein.

Diffusion. A Tiselius apparatus equipped with a diagonal-edge schlieren system was used at a constant temperature of 0·5°. The boundaries were sharpened by Kahn & Polson's (1947) method. Because of the rapid aggregation of cod myosin, readings were taken only during the first 24 hr.; after this time progressively lower values for the diffusion coefficient were obtained. The rate of diffusion was so slow and the concentration of protein so low that considerable difficulties were experienced in obtaining boundaries which remained undistorted during the first 24 hr. Less than half the experiments proved successful and it is therefore difficult to be really confident of the accuracy of the remainder. The diffusion coefficient was calculated by the maximum-ordinate-area method.

The diffusion coefficient has been also computed from one velocity ultracentrifuge run with Baldwin's (1957) treatment of Fujita's (1956) equation. In this the height-area ratio of the peaks in successive photographs is corrected for the sharpening effect produced on the diffusion gradient of molecules whose sedimentation is concentra-

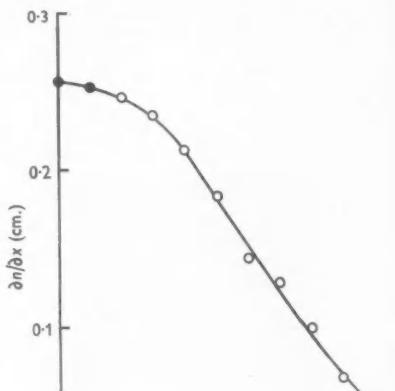

Fig. 3. Method of extrapolating concentration-gradient curves in the Archibald procedure. $\partial n/\partial x$ is the value of the concentration gradient (actually refractive-index gradient) measured in cm. at the photographic plate. ○, Experimental points; ●, extrapolated points.

tion-dependent, and this ratio squared, $[(A_0/H).F(\xi_m)]^2$, is plotted against $(e^\tau - 1)\,[1+(1-\lambda)^{\frac{1}{2}}]^2$, where A_0 is the area of the peak corrected for dilution during sedimentation in a sector-shaped cell, H is the height of the peak, $F(\xi_m)$ is a function incorporating the concentration-dependence of sedimentation, and τ and λ are terms incorporating the elapsed time from the start of the experiment. For homogeneous solutes this plot is linear and the slope is $2D/\omega^2 S_0$, where D is the diffusion coefficient of the solute at the temperature and solute concentration of the experiment, ω is the angular velocity and S_0 is the sedimentation coefficient of the solute at infinite dilution and at the temperature of the experiment. In the present experiment the sedimentation coefficient at infinite dilution and the concentration-dependence of sedimentation found for cod myosin (see below) have been used in the calculation.

Viscosity. An Ostwald viscometer (B.S. 188:1957, no. 1) held at 0·5° has been used. The efflux time with buffer was 449 sec. and the rate of shear calculated by Kroepelin's (1929) formula varied between 454 and 272 sec.$^{-1}$ for the buffer and most concentrated protein solution respectively. The kinetic-energy correction was negligible.

Turbidity measurements. A Unicam SP. 500 spectrophotometer was used with matched 4 cm. cells. Dialysed cod-actomyosin solutions in 0·5 M-KCl, pH 6·3, were centrifuged at 20 000 *g* until the turbidity was constant, and samples of the supernatant solution were diluted with 0·5 M-KCl, quickly warmed to room temperature and their optical densities determined at selected wavelengths between 361·1 and 670·8 mμ. The mean temperature of the solutions at the beginning and end of the readings was used in the computation of refractive index. Protein concentrations ranged from 0·05 to 0·3%. The refractive index of the solvent was calculated from the values given for water in the International Critical Tables, vol. VII (taking account of wavelength and temperature), and the average specific-refractive-index increment of 0·5 M-KCl was determined with a dipping refractometer (0·00460). The refractive-index increment of cod actomyosin was assumed to be the same as that of rabbit actomyosin (Harkness & Wassermann, 1954). M was calculated according to Harkness & Wassermann (1954) from the reciprocal intercept of plots of $(Hc/\tau)_{c\to 0}$ against $(n_0/\lambda)^2$, where τ/c is the reduced turbidity and H is a factor incorporating the wavelength (λ), the refractive-index increment of actomyosin and the refractive index of the solvent (n_0).

Optical rotation. An instrument reading to 0·01° was used. Observations were made at a temperature of 23·5±0·5° in 4 dm. tubes with sodium light.

Partial specific volume. Determinations were made in 10 ml. density bottles at 20·0°. The myosin solutions (concentration about 0·35%) were dialysed beforehand at 0° for 4 days against several changes of KCl–phosphate buffer ($I = 0{\cdot}5$).

Protein concentration. Usually micro-Kjeldahl determinations of nitrogen were used; sometimes they were supplemented by gravimetric determinations of the protein insoluble in 10% trichloroacetic acid. The nitrogen content of dry, ash-free cod myosin was found to be 16·4%; those of actin and actomyosin were assumed to be both 16·0%.

RESULTS

Sedimentation of cod myosin. Within the temperature range of 0–7° the values of the corrected sedimentation coefficient (Fig. 4) are consistent, and show no systematic variation with temperature, type of muscle (frozen or fresh) or type of dissociating reagent (ATP or pyrophosphate). Each point represents one distinct preparation. Application of the method of least squares leads to the following values ($S = S_0 - kc$): $S_0 = 6{\cdot}43 \times 10^{-13}$ (standard error $0{\cdot}08 \times 10^{-13}$), $k = 3{\cdot}18 \times 10^{-13}$ (standard deviation $0{\cdot}39 \times 10^{-13}$), correlation coefficient 0·89. c is the initial protein concentration in g./100 ml. A single determination of the sedimentation coefficient of rabbit myosin prepared by diluting an extract of rabbit muscle gave a value in KCl–phosphate buffer ($I = 0{\cdot}5$) (Fig. 4) which did not differ significantly from that of cod myosin at the same concentration.

Two runs with monodisperse cod myosin at temperatures of 10·8° and 13·4° and protein concentrations of 0·32 and 0·51% gave respectively values of $5{\cdot}60$ and $5{\cdot}31 \times 10^{-13}$ for the sedimentation coefficient. These values are significantly different from the values at the same concentration expected from Fig. 4, the first barely so. A run at 21·8° with cod myosin which was monodisperse at 5° showed several small peaks, the sedimentation coefficient of the slowest being 6·60. Presumably aggregation occurred at this relatively high temperature during the period of approximately 30 min. before starting the acceleration of the rotor. The concentration of the protein corresponding to the slowest peak could not be measured accurately but was probably about 0·05 g./100 ml. These results at high temperatures indicate that the corrected sedimentation coefficient of cod myosin is temperature-dependent, as appears to be the case with

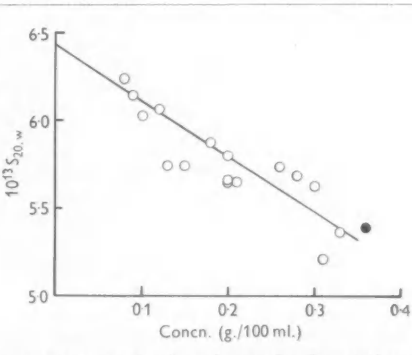

Fig. 4. Concentration-dependence of sedimentation coefficient, ○, of cod myosin; ●, of rabbit myosin.

rabbit myosin (Laki & Carrol, 1955; Parrish & Mommaerts, 1954).

Diffusion of cod myosin. The diffusion coefficient of cod myosin was found to vary very little with concentration between 0·19 and 0·40 g./100 ml., the mean value of six determinations being $1{\cdot}10 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, standard error $0{\cdot}05 \times 10^{-7}$. The shape of the diffusion curve of the final photograph of one run (Fig. 5) deviated from ideality in showing considerable sharpening but no skewing.

The results of applying Fujita's (1956) equation to one sedimentation experiment at a myosin concentration of 0·33 g./100 ml. are shown in Figs. 6 and 7. The plot of $[(A_0/H).F(\xi_m)]^2$ against

$$(e^{\tau}-1)\,[1+(1-\lambda)^{\frac{1}{2}}]^2$$

shows an upward curvature indicating that the preparation is heterogeneous. The magnitude of the correction for the sharpening effect with this protein is shown by the plot of $[A_0/H.1{\cdot}128]^2$ against $(e^{\tau}-1)\,[1+(1-\lambda)^{\frac{1}{2}}]^2$, 1·128 being the value $(2/\sqrt{\pi})$ assumed by $F(\xi_m)$ when the sedimentation of the solute is not concentration-dependent. Apparent diffusion coefficients, D^*, (Fig. 7), have been calculated from the data of Fig. 6 assuming that the curves pass through the origin. The value of the diffusion coefficient at zero time, after correction to 20°, is about $1{\cdot}2 \times 10^{-7}$ cm.^{2} sec.$^{-1}$.

Partial specific volume of cod myosin. A value of 0·735 at 20° and a mean concentration of 0·34 g./100 ml. was found with Kraemer's equation 115 (Svedberg & Pedersen, 1940). The value at infinite dilution is probably slightly less than this, which brings it into close agreement with Parrish & Mommaerts' (1954) value of 0·728 for rabbit myosin at 26°. A value of 0·730 at 20° has been used in the calculations of sedimentation coefficient and molecular weight.

Molecular-weight and frictional ratio of cod myosin from sedimentation and diffusion results. Insertion of the values for sedimentation coefficient and diffusion coefficient, determined from a stationary boundary, in the Svedberg equation gives a value of 530 000 for the molecular weight with a standard deviation of about 30 000. The frictional coefficient is 3·62. From Perrin's (1936) equation for prolate

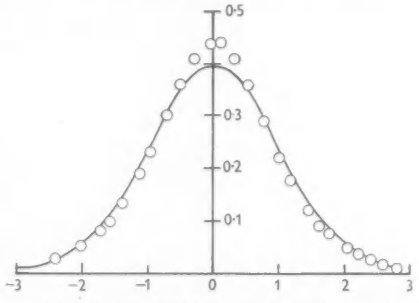

Fig. 5. Normalized diffusion curve of cod myosin. ○, Experimental points of the diffusion curve in normalized co-ordinates. The full line is the normal curve; 94 140 sec. after the start of the experiment; concentration 0·40 g./100 ml.

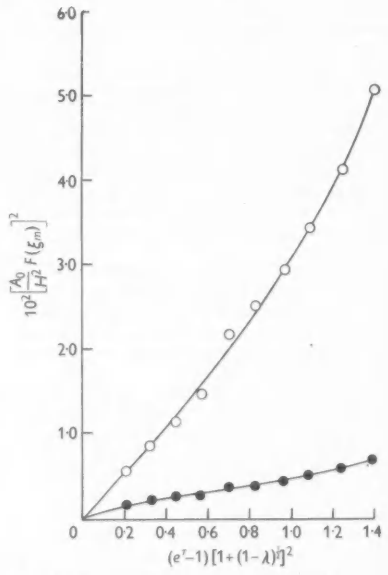

Fig. 6. Height–area plots of the concentration-gradient curve of cod myosin in an ultracentrifuge run at 42 040 rev./min. ○, Plot corrected, and ●, uncorrected, for concentration-dependence of sedimentation. Initial concentration 0·33 g./100 ml.

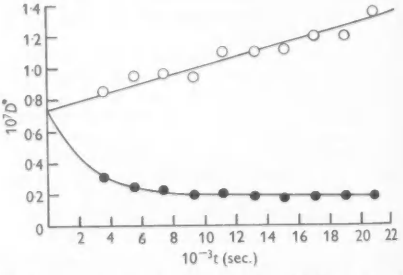

Fig. 7. Apparent diffusion coefficients of cod myosin, D^*, calculated from the points of Fig. 6. ○, Corrected, and ●, uncorrected, for concentration-dependence of sedimentation

Table 1. *Molecular weight of a cod-myosin preparation calculated from successive exposures of an Archibald experiment*

Temperature 1·40°; concentration 0·44 g./100 ml.; 9230 rev./min.

Time after reaching full speed (min.)	33	65	97	129	151
Molecular weight/10^5	5·5	5·4	6·4	5·9	5·4

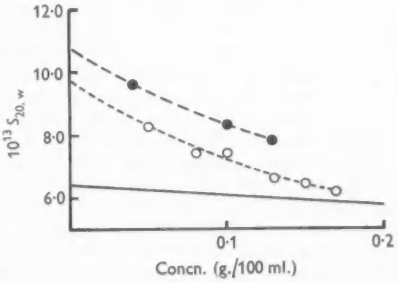

Fig. 8. Concentration-dependence of sedimentation of cod-myosin aggregates. ○, Dimer; ●, trimer. Full line is taken from Fig. 4 and refers to myosin.

ellipsoids of revolution, the latter corresponds to axial ratios of about 70 and 60 assuming 10 and 30 % of hydration respectively.

Molecular weight of cod myosin from Archibald procedure. The mean of four determinations at temperatures of 1·4–6·4° and concentrations of 0·23–0·44 g./100 ml. was 590 000, standard deviation 50 000. The molecular weight calculated from successive exposures (Table 1) showed no trend with time, indicating that the preparations were essentially monodisperse.

Intrinsic viscosity of cod myosin. Values of $\eta_{sp.}/c$ were plotted against c for four preparations, and extrapolated to infinite dilution to give an intrinsic viscosity of 1·80, standard deviation 0·05. $\eta_{sp.}$ and c are the specific viscosity and concentration in g./100 ml. respectively. This value corresponds to axial ratios of 58 and 50 for prolate ellipsoids of revolution with 10 and 30 % of hydration respectively (Simha, 1940).

Optical rotation of cod myosin. One preparation had $[\alpha]_D^{23} = -29°$ in KCl–phosphate buffer ($I = 0·5$, protein concentration 0·32 g./100 ml.) and $-104°$ in KCl–phosphate buffer ($I = 0·5$) made up to 8 M-urea (protein concentration 0·17 g./100 ml.) respectively. These results remained constant for 24 hr., the solutions being kept at 0°.

Aggregation of cod myosin. The process of aggregation appears to be a much more rapid one than with rabbit myosin. Amounts of oligomers estimated at about 5–10 % of the total protein content are present in cod-myosin solutions kept at 0° for 18–20 hr., whereas solutions of rabbit myosin can be kept unchanged for several days under the same conditions. Approximate sedimentation coefficients of the two main peaks which sediment more rapidly than monodisperse myosin are shown in Fig. 8. At infinite dilution the values are about $9·0–9·5 \times 10^{-13}$ and $10·5–11·0 \times 10^{-13}$, which suggests that they are respectively the dimer and trimer of a side-to-side myosin-aggregation process. For very elongated molecules, the sedimentation coefficients of the side-to-side dimer and trimer are respectively about $\sqrt{2}$ and $\sqrt{3}$ times that of the monomer (Weber & Portzehl, 1952*b*); for myosin this would give values of about $9·1 \times 10^{-13}$ and $11·0 \times 10^{-13}$ for the dimer and trimer respectively. The type of aggregation therefore appears to resemble that undergone by rabbit myosin.

The aggregation does not appear to be dependent upon the presence in the molecule of intact sulphydryl groups (at least those that are freely reactive), because samples of monodisperse cod myosin which were treated with *N*-ethylmaleimide until they no longer gave a nitroprusside reaction aggregated as quickly as untreated samples.

Aggregation is promoted by diluting solutions of cod myosin until the protein precipitates. This effect, first noted with extracts (Connell, 1958), also occurs with solutions of monodisperse cod myosin. It is not prevented by precipitating the protein into and redissolving it in solutions containing either 0·01 M-sodium cysteinate or 0·01 M-ethylenediaminetetra-acetic acid at pH 7. Ultracentrifugal analysis of a mixture of cod myosin and its aggregates to which 3 M-urea had been added immediately before showed a main component sedimenting at a rate much slower than that of myosin, and two or three smaller components sedimenting at rates similar to those of myosin and its aggregates at similar concentration. Apparently urea at this concentration, in contrast with its effect on aggregated fibrinogen (Caspary & Kekwick, 1957), disaggregates cod myosin into fragments smaller than the monomer. Urea at concentrations of 6–7 M is known to dissociate rabbit-myosin monomer but at a relatively slower rate (Snellman & Erdös, 1948; Tsao, 1953).

It was shown that cod myosin is definitely more sensitive than rabbit myosin to precipitation by extracting simultaneously, and with the same batch of solution, muscle removed from recently killed cod and rabbit and working up the extracts (see Experimental section) exactly in parallel. The cod preparation showed in the ultracentrifuge the

presence of much dimer and trimer, but the rabbit myosin remained perfectly homogeneous throughout the run even though it was examined 3 hr. after the cod myosin. Various authors have noted a tendency of rabbit myosin to aggregate on repeated precipitation (Snellman & Erdös, 1948; Portzehl, Schramm & Weber, 1950).

Sedimentation coefficient, molecular weight and frictional ratio of cod G-actin. The sedimentation coefficients of a limited number of purified cod G-actin preparations in a number of solvents are given in Fig. 9. The protein remaining in the supernatant solution of runs containing KCl ($I = 0{\cdot}3$) is presumably inactive actin, i.e. actin no longer able to polymerize; these preparations were obtained by prolonged dialysis against KCl ($I = 0{\cdot}3$). The values of the sedimentation coefficient determined in water are susceptible to serious errors due to charge effects but do not appear to be very much out of line with the values obtained in the presence of salt (cf. Mommaerts, 1952). The extrapolated sedimentation coefficient is about $3{\cdot}3 \times 10^{-13}$, which taken together with the diffusion coefficient of cod G-actin (Connell, 1954), $2{\cdot}3 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, gives a molecular weight of about 130 000 and frictional ratio of 2·7. Assuming 30 % of hydration, the latter value corresponds to an axial ratio of 25 for a prolate ellipsoid of revolution.

Molecular weight of cod actomyosin. Turbidity measurements on three preparations gave figures in the region of 6, 6 and 8×10^7 for the molecular weight of cod actomyosin.

DISCUSSION

There are so many divergencies in the data for myosins prepared by different laboratories that it is useful to summarize the results if comparison between cod and other animal myosins is to be made (Table 2).

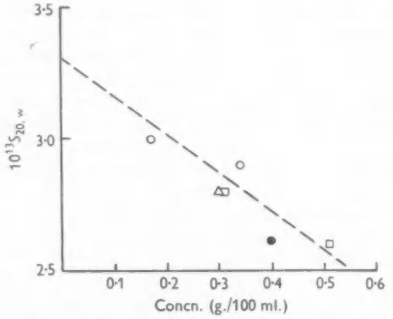

Fig. 9. Concentration-dependence of sedimentation of ultracentrifugally purified cod G-actin. ○, 0·1 mM-ATP, pH 8·5; □, KCl–phosphate, pH 7·5 ($I = 0{\cdot}3$); △, KI–phosphate, pH 7·5 ($I = 0{\cdot}4$); ●, KI–phosphate, pH 7·5 ($I = 0{\cdot}66$).

The group of sedimentation coefficients above 7×10^{-13} were all obtained with either oil- or air-turbine ultracentrifuges, and the remaining, significantly lower group, all with Spinco machines; it is likely that part of the discrepancy between the two groups is traceable to differences in rotor-temperature calibration (Edsall, 1953). The scatter in the values obtained with Spinco machines is perhaps no greater than that for some other proteins when they are examined in different laboratories, but it also seems that the sedimentation behaviour of myosin is complicated by two different effects: aggregation and the effect of temperature on the frictional coefficient of the myosin monomer. Most authors describe myosin as a simple, sharp peak in the ultracentrifuge and it is not likely therefore that the variations in sedimentation coefficients are the result of a side-to-side type of aggregation (Holtzer, 1956; Holtzer & Lowey, 1956), since the oligomers formed are easily separable from the monomer by virtue of their much higher sedimentation coefficients ($> 9 \times 10^{-13}$). End-to-end aggregation would yield oligomers with sedimentation coefficients only slightly different from that of the monomer, and it is possible that some variability in sedimentation behaviour as well as the heterogeneity of cod myosin observed in the present work may be ascribed to this phenomenon. It is a reasonably well authenticated fact that the sedimentation coefficient of monodisperse myosin, even after correction in the usual way, shows a positive temperature-dependence (Blum & Morales, 1953; Parrish & Mommaerts, 1954; Laki & Carrol, 1955). This could be explained either by an end-to-end aggregation process whose equilibrium position, like that of side-to-side aggregation, is temperature-dependent, or by a variation of the frictional coefficient of the molecule with temperature. In either case it is possible to obtain a correct value of the average molecular weight from the sedimentation results by combining the data, as in the present work, with results obtained from diffusion experiments made at the same temperature.

Parrish & Mommaerts (1954) suggest that the corrected sedimentation coefficient of rabbit myosin may be dependent upon the angular velocity of the rotor. In the present work two determinations at 42 040 rev./min. of the sedimentation coefficient of cod myosin did not differ significantly from the bulk of the results, which were obtained at 59 780 rev./min.

In view of the variability of S_0 for myosin it is hardly surprising that large differences are found for the concentration-dependence of sedimentation. That for cod myosin is, however, not out of line

with the results for rabbit myosin. The low value of k obtained for carp myosin (Hamoir, 1951, 1955*a*, *b*) is probably the result of using the concentration of actomyosin from which the myosin was derived by dissociation rather than the concentration of myosin itself.

The diffusion coefficient of cod myosin agrees well with latest estimates for rabbit myosin. The absence of any concentration-dependence is surprising for such an asymmetric molecule as cod myosin, and it is suspected that the results at concentrations below 0·2 g./100 ml. are too low; a similar suggestion has been made for rabbit myosin (Laki & Carrol, 1955). On the other hand, the extremely large dependence on concentration of the diffusion coefficient of rabbit myosin found by Portzehl *et al.* (1950) is difficult to reconcile with the fact that the concentration-dependence of diffusion should be less than that of sedimentation (Singer, 1947).

The molecular weight of cod myosin both from sedimentation and diffusion and from the Archibald procedure are in good agreement with the latest results on rabbit myosin with sedimentation–diffusion (Laki & Carrol, 1955) and light-scattering (Rupp & Mommaerts, 1957; Holtzer, 1956). The same is true of the axial ratio of cod myosin calculated from the frictional coefficient, though the axial ratio calculated from the intrinsic viscosity is slightly lower. Low values of the axial ratio calculated from viscosity data in contrast with those calculated from sedimentation and diffusion data are also obtained for some other very asymmetric proteins, e.g. tropomyosin and fibrinogen. (Since this paper was prepared two groups of workers have obtained a molecular weight of about 420 000 for rabbit myosin with the Archibald procedure.)

It is not possible to attribute the marked instability of cod myosin to some peculiarity of its molecular weight or general shape. The structural similarity between the cod and rabbit proteins appears to extend to the configuration of the secondary structure of their molecules, since the optical rotation of cod myosin is almost the same as that given for rabbit at the same wavelength

Table 2. *Collected data on the skeletal-muscle myosin of various animals*

Animal	$10^{13} \times S_{20,w}$*	$10^{13} \times k$†	$10^{7} \times D_{20,w}$*	$10^{-5} \times M$‡	$[\eta]$§ (100 ml. g.$^{-1}$)	Reference
Rabbit	—	—	—	—	2·5	1, 2
Rabbit	7·2 (20)	3·3	0·5 (15)	15 (SD)	—	3
Rabbit	7·1 (20)	3·3	0·87–0·90 (20)	8·29–8·88‖ (SD) 8·4 (O)	2·2	4
Rabbit	7·1 (20)	5·2	—	—	—	5
Rabbit, rat, mouse	6·2 (7)	5·0	—	—	2·0	6
Rabbit	6·66–6·85 (23·6)	5·21	1·05 (1·1)	—	—	7
Rabbit	6·15 (4)	2·6	1·0 (1·0)	5·0¶ (SD)	—	8
Rabbit	6·38–6·43 (25)	—	—	5·3 (LS)	2·2–2·4	9
Carp	6·55 (20)	1·6	—	—	—	10
Frog	6·6 (20)	—	—	—	2·0 (rabbit)	11
Rabbit	—	—	—	—	1·7	12
Rabbit	—	—	—	6·5** (LS)	—	13
Cod	6·45 (0–5)	3·18	1·10 (0·5)	5·3 (SD) 5·9 (A)	1·80 —	14 —

* Sedimentation and diffusion coefficients at infinite dilution; figures in parentheses are temp. of measurement.

† Values in the equation: $S = S_0 - kc$ (c in g./100 ml.). Where k is not stated explicitly in the reference it has been estimated from the published figures.

‡ Calc. from sedimentation and diffusion (SD), osmometry (O), light-scattering (LS) and Archibald procedure (A).

§ Intrinsic viscosity, values from references 4, 11 and 12, are viscosity number (Portzehl, Schramm & Weber, 1950) × 10, which is only an approximate conversion.

‖ If $V = 0{\cdot}73$ is used, these values are nearer 7·7–8·0.

¶ Value given in reference 8; correct value appears to be 5·5.

** Provisional estimate; correct value may be lower.

References to Table 2

1. Guba & Straub (1943).
2. Mommaerts (1945).
3. Snellman & Erdös (1948).
4. Portzehl *et al.* (1950).
5. Johnson & Landolt (1951).
6. Miller, Golder, Eitelman & Miller (1952)
7. Parrish & Mommaerts (1954).
8. Laki & Carrol (1955).
9. Holtzer & Lowey (1956).
10. Hamoir (1951).
11. Hamoir & Reuter (1956).
12. Kerekjato (1952).
13. Rupp & Mommaerts (1957).
14. Present work.

(Cohen & Szent-Györgyi, 1957). The explanation for the readiness with which cod myosin aggregates probably lies rather in some idiosyncrasy of molecular topography or reactivity of surface groups. Certain soya-bean-protein fractions aggregate in a similar way to cod myosin when their solutions are diluted, and the interaction of sulphydryl groups has been shown to be involved in the process (Briggs & Wolf, 1957). What evidence there is suggests that the aggregation of cod myosin is not of this kind; it has been also noted (J. J. Connell, unpublished work) that the sulphydryl content of cod myosin appears to be not very different from that of rabbit myosin.

The order of the molecular weight of cod actomyosin determined by turbidity is about one-half or one-third of the values obtained for rabbit actomyosin with the same method (Harkness & Wassermann, 1954), but very similar to those for rabbit actomyosin from light-scattering measurements (Blum & Morales, 1953) or for carp actomyosin from sedimentation and viscosity measurements (Hamoir, 1955*a*).

The value of the sedimentation coefficient of cod G-actin and the resulting value for molecular weight, when combined with the diffusion coefficients, substantiate the earlier observations (Connell, 1954) that this protein closely resembles rabbit G-actin and in particular agree with the conclusion from osmotic-pressure measurements that it exists mainly in the form of the dimer of molecular weight about 150 000. The value for the axial ratio of cod G-actin estimated from sedimentation and diffusion also agrees with Tsao's (1953) viscometric value for the rabbit-protein dimer, though in view of the approximate nature of the data this agreement is perhaps fortuitous.

SUMMARY

1. Solutions of unaggregated cod myosin have been prepared by ultracentrifugal separation after dissociation of cod actomyosin with adenosine triphosphate or pyrophosphate.

2. The sedimentation and diffusion coefficients of cod myosin at infinite dilution have been measured and the molecular weight and shape calculated from these data with the measured partial specific volume.

3. Investigation of the diffusion behaviour both in stationary boundaries and in boundaries in the ultracentrifuge indicate that the preparations of cod myosin are heterogeneous.

4. The molecular weight of cod myosin has been also determined by the Archibald procedure and the axial ratio assessed from the intrinsic viscosity.

5. Some aspects of the aggregation of cod myosin have been examined.

6. Cod actin has been purified in the ultracentrifuge and its sedimentation coefficient determined.

7. The order of magnitude of the molecular weight of cod actomyosin has been determined from turbidity measurements.

8. The size and shape of the cod fibrillar proteins are very similar to those of the rabbit proteins. The unusual properties of the fish proteins cannot be explained therefore on this basis.

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The Changes in the Total Vitamin B_6 and the Pyridoxal Phosphate Content of Cells of *Bacillus sphaericus* during Growth and Sporulation: their Possible Relationships with αε-Diaminopimelic Acid Metabolism

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The present study was prompted by the observation that vegetative cells of *Bacillus sphaericus* contained no αε-diaminopimelic acid (DAP), whereas sporulating cells and spores contained 1–2% (w/w) of DAP (Powell & Strange, 1957). A connexion between DAP metabolism and sporulation was already suspected: from structural considerations and the analogous conversion of lysine into piperidine-2-carboxylic acid (Rothstein & Miller, 1953; Lowy, 1953) it seemed possible that DAP was the precursor of pyridine-2:6-dicarboxylic acid (dipicolinic acid), a substance synthesized in large amounts by sporulating cells (Powell, 1953; Powell & Strange, 1956). In other *Bacillus* species there was, however, no connexion between sporulation and the appearance of DAP as a cell constituent: vegatative cells, sporulating cells and spores all contained approximately equal amounts of DAP. *B. sphaericus* was also exceptional among *Bacillus* species (Antia, Hoare & Work, 1957) in possessing an active DAP decarboxylase system requiring pyridoxal phosphate as a cofactor (Powell & Strange, 1957). A comparison of the DAP decarboxylase activities of vegetative cells, sporulating cells and spores showed that although the decarboxylase activity of *B. sphaericus* cultures decreased at sporulation, the activity of strongly sporulating cells was still 50% of that of vegetative cells. There was therefore no sudden disappearance of the decarboxylase at sporulation to account for the appearance of DAP; nor was its activity in disrupted cells inhibited by dipicolinic acid (Powell & Strange, 1957). There remained the further possibilities that (*a*) other unidentified substances synthesized during sporulation inhibited the decarboxylase or (*b*) that sporulation was associated with a disturbance of total vitamin B_6 or, more particularly, of pyridoxal phosphate synthesis. The present report is chiefly concerned with the latter possibilities and also includes some data on DAP decarboxylase and vitamin B_6 synthesis during spore germination and development.

EXPERIMENTAL

Materials

Organisms. Most of the work was done with *B. sphaericus*, National Collection of Type Cultures (N.C.T.C.) 9602, since this strain usually sporulated strongly and gave 'clean' spore suspensions, i.e. suspensions containing very little vegetative debris, after growth for 24 hr. in the test medium. A few observations were made on a laboratory strain of *Bacillus cereus* which was used in a previous study (Powell & Strange, 1956). Some growth tests were made with *B. sphaericus* N.C.T.C. 7582 var. *fusiformis*, an asporogenous variant of *B. sphaericus* N.C.T.C. 7582 (Powell & Hunter, 1955) and a laboratory strain of *B. sphaericus* var. *rotans*.

Chemicals. Pyridoxal hydrochloride and pyridoxine hydrochloride were supplied by L. Light and Co., pyridoxal phosphate by S. A. F. Hofmann–La Roche and Co., deoxypyridoxine hydrochloride by Nutritional Biochemical Corp. and *iso*nicotinylhydrazine by Ward, Blenkinsop and Co.

Methods

Assays for total vitamin B_6 and pyridoxal phosphate. Cells of *B. sphaericus* N.C.T.C. 9602 and of a laboratory strain of *B. cereus* at various stages of growth and sporulation were produced in an enriched potato-extract medium as previously described (Powell & Strange, 1956, 1957). The cells were harvested by centrifuging, washed once with water and assayed for total vitamin B_6 by an unpublished modification of the method of Atkin, Schultz, Williams & Frey (1943). The modified method was developed in the Department of Biochemistry, Oxford, and was made available through the courtesy of Professor D. D. Woods.

Washed cells of *B. sphaericus* were assayed for pyridoxal phosphate with the manometric method of Umbreit, Bellamy & Gunsalus (1945). The cells were reaped, washed, and mechanically disintegrated for 45–60 min. at 4° (Mickle, 1948) and assayed on the same day. To reduce the risk of pyridoxal phosphate losses during disintegration the cells were suspended (1–2 mg. dry wt./ml.) in 0·2 M-acetate buffer, pH 5·5, and the glass containers were wrapped with black paper. The reaction mixture for the assay contained 2 mg. (dry wt.) of *Streptococcus faecalis* cells, 50–200 μg. (dry wt.) of disintegrated cells of *B. sphaericus* and 50 μ-moles of finely ground tyrosine in a total volume of 2·7 ml. of 80 mM-acetate buffer, pH 5·5. The manometers were filled with nitrogen and shaken at 30°. Cells of *B. sphaericus* from cultures at various stages of growth and sporulation were assayed simultaneously and at two cell concentrations. A set of calibration flasks containing 0, 2, 4, 6 and 8 μmg. of pyridoxal phosphate instead of *B. sphaericus* cells was included in each experiment.

The DAP decarboxylase activity of freshly reaped disintegrated cells of *B. sphaericus* was assayed manometrically with unresolved synthetic DAP as substrate as previously described (Dewey, Hoare & Work, 1954; Powell & Strange, 1957).

Determination of the isomeric constitution of the diaminopimelic acid present in sporulating cells. Freeze-dried sporulating cells of *B. sphaericus* N.C.T.C. 9602 (20 mg.) were hydrolysed in 0·5 ml. of 6 N-HCl at 105° for 24 hr. After removal of the HCl *in vacuo*, the hydrolysate was applied as a band on to Whatman no. 4 paper and run with collidine–lutidine–water (60:15:75, by vol.) for 18 hr. A paper spotted with synthetic DAP was run in parallel. The dried papers were examined in ultraviolet light and fluorescent bands in corresponding positions to those taken up by the synthetic DAP were cut out and eluted (Powell & Strange, 1956). A volume of eluate corresponding to 2 mg. of cells was spotted on to Whatman no. 1 paper and run for 18 hr. in pyridine–methanol–conc. HCl–water (10:80:2·5:17·5, by vol.) (Rhuland, Work, Denman & Hoare, 1955). Comparison with reference spots of the DAP isomers (Rhuland *et al.* 1955) indicated that the *meso*- or D-isomer or both were present in sporulating cells of *B. sphaericus*. It was possible to distinguish between the *meso*- and D-isomers by treatment with DAP decarboxylase, which attacks only the *meso*-isomer (Hoare & Work, 1955). The remainder of the eluate from the collidine chromatogram, containing approx. 180 μg. of DAP, was distributed equally among three small tubes. To one of these tubes, 40 μg. of *meso*-DAP was also added and to another, 40 μg. of D-DAP. All three tubes were then evaporated to dryness *in vacuo*. Equal amounts (0·2 ml.) of a suspension of disintegrated vegetative cells of *B. sphaericus* (10 mg. dry wt. of cells/ml.) of high DAP decarboxylase activity were added to each tube, together with 5 μg. of pyridoxal phosphate. The tubes were incubated at 37° for 1 hr. Ethanol (0·2 ml.) was added to each tube and, after centrifuging, samples (0·2 ml.) of the supernatants were spotted on to Whatman no. 1 paper and run in the solvent system of Rhuland *et al.* (1955) for 18 hr. No trace of DAP was detected in the portions of eluate which had no addition or an addition of *meso*-DAP, whereas the portion of eluate to which D-DAP was added gave a strong DAP spot. It therefore appeared that sporulating cells of *B. sphaericus* contained only the *meso*-isomer of DAP.

RESULTS

The total vitamin B_6 content of *B. sphaericus* cells was taken as the mean value from assays at two levels of cell concentration each carried out in duplicate. The mean coefficient of variation within 83 duplicates, which included pyridoxine standards, was 0·032. The mean coefficient of variation between the means of duplicates at two levels of cell concentration was 0·082. The pyridoxal phosphate content of *B. sphaericus* cells was taken as the mean value from single assays at two levels of cell concentration. The mean coefficient of variation between assays at two levels of cell concentration was 0·102. The changes found to occur in the total vitamin B_6 and pyridoxal phosphate content of *B. sphaericus* cells at various stages of growth and sporulation were considerably greater than could be accounted for by experimental error. A relatively small number of vitamin B_6 assays were made in the same way on cells of a laboratory strain of *B. cereus*. The variation between duplicates and between assays at two levels of cell concentration were well within the limits of variation found for *B. sphaericus*.

Sporulation

The results of four separate experiments in which the vitamin B_6 content of vegetative cells, sporulating cells and free spores of *B. sphaericus* was determined are given in Table 1. In Expts. 3 and 4, values for pyridoxal phosphate content are also included. From these experiments, and from scattered observations on cells from cultures of various ages, it was concluded that the total vitamin B_6 and the pyridoxal phosphate content of the cell did not decrease and in some cultures even increased during sporulation, i.e. during the period 13–18 hr. after inoculation while the spore was developing inside the sporangium. Vitamin B_6 assays on vegetative and sporulating cells gave values in the range 300–550 μm-moles/g. dry wt. The pyridoxal phosphate content of vegetative and sporulating cells range between 180 and 330 μm-moles/g. In instances where determinations of the total vitamin B_6 and pyridoxal phosphate

Table 1. *Total vitamin B_6 and pyridoxal phosphate content of vegetative cells, sporulating cells and free spores of* Bacillus sphaericus *N.C.T.C.* 9602

Age of culture (hr.)	Appearance of culture	Vitamin content (μm-moles/g. dry wt. of cells) Expt. 1 Vitamin B_6	Expt. 2 Vitamin B_6	Expt. 3 Vitamin B_6	Expt. 3 Pyridoxal phosphate	Expt. 4 Vitamin B_6	Expt. 4 Pyridoxal phosphate
9	Vegetative cells	480	532	408	227	490	204
16	Strongly sporulating cells	404	550	396	265	580	330
20	Sporulation complete: approximately one-third of spores still enclosed by sporangia	160	—	—	—	—	—
24	Free spores*	101	200	83	72	403*	212*

* Sporulation was incomplete in the 24 hr. culture of Expt. 4 and there were very few free spores.

Table 2. *Total vitamin B_6 content of vegetative cells, sporulating cells and free spores of a laboratory strain of* Bacillus cereus

Age of culture (hr.)	Appearance of culture	Vitamin B_6 content (μm-moles/g. dry wt. of cells) Expt. 1	Expt. 2
10	Vegetative cells	162	224
17	Strongly sporulating cells	150	145
24	Mainly free spores	99	73

content of the same batch of cells were made, the pyridoxal phosphate represented 40–60 % of the total vitamin B_6 content. In 24 hr. cultures, which consisted mainly of free spores, the vitamin B_6 and pyridoxal phosphate content of the harvested cells had fallen to, at most, 50 % and usually to 25 % of that of vegetative and sporulating cells. Low values for cells from 24 hr. cultures were obtained only when sporulation had proceeded to completion and the spores were mainly free of sporangial material. In Expt. 4 (Table 1), although growth appeared to have proceeded normally, sporulation was incomplete at 24 hr. and there were very few free spores. The vitamin B_6 content of cells from this culture at 24 hr. was only slightly lower than that at 9 and 16 hr. Similarly, in Expt. 1 (Table 1) at 20 hr., the culture had sporulated well, but approximately one-third of the spores were still enclosed by sporangia: a higher value for the vitamin B_6 content was obtained at 20 hr. than at 24 hr. when all the spores were free. It was thus possible to predict the approximate vitamin B_6 content of harvested cells from a 24 hr. culture by an examination of stained films (cf. plate 1, Powell & Strange, 1957).

The total vitamin B_6 and pyridoxal phosphate content of washed-spore suspensions did not appear to change appreciably during storage at 0° for 4–8 weeks. Vitamin B_6 assays made on intact and on mechanically disintegrated resting spores gave values which were similar to each other and to those obtained with germinated spores (see below). It therefore seemed unlikely that the low values obtained for the vitamin B_6 content of resting spores could be attributed to the difficulties of extraction associated with their peculiar structure.

The results of vitamin B_6 assays on vegetative cells, sporulating cells and free spores of a laboratory strain of *B. cereus* are given in Table 2. Two cultures of *B. cereus* were examined, and in both cases the vitamin B_6 content of spores was considerably lower than that of vegetative cells. In one culture, sporulating cells reaped after growth for 17 hr. contained significantly less vitamin B_6 than vegetative cells reaped after growth for 10 hr., whereas in the other culture sporulating cells and vegetative cells contained approximately equal amounts of vitamin B_6.

Spore germination and development

The changes in the total vitamin B_6 content of spores of *B. sphaericus* during germination and growth were also studied. Resting-spore suspensions were heated at 60° for 1 hr. to stimulate subsequent germination (Curran & Evans, 1945; Powell, 1950) and added to the enriched potato-extract medium at 37° to give suspensions containing 0·2–1·0 mg. dry wt. of spores/ml. The suspensions were then shaken at 37°. Samples were taken out at various intervals, cooled to 0° and centrifuged and the cells washed once with water before being assayed for vitamin B_6. Stained films indicated that during the first 1 hr. germination was practically complete (Powell, 1950), and there was some swelling of the germinated forms. Development and growth of the first vegetative cells occurred during the following 2 hr. No sign of cell division was seen in these suspensions, and it was noticed that the developing vegetative cells contained one or two terminal volutin granules, in contrast with the uniformly staining, featureless vegetative cells of 6–8 hr. cultures growing from a

small inoculum of 0·05–0·10 μg. dry wt. of spores/ml. The results of these experiments with developing spores are summarized in Table 3. In every experiment synthesis of vitamin B_6 occurred during the first hour of development. This was especially marked in the more dilute suspensions inoculated with 0·2 mg. dry wt. of spores/ml.: here there was an approximately threefold increase in the cellular vitamin B_6. From scattered assays made after incubation for 2 and 3 hr. it appeared that there was a further slight increase in the vitamin B_6 content of the developing vegetative cells, followed by a decrease. The highest values obtained were 200–220 μm-moles of vitamin B_6/g. dry wt. of cells, corresponding to approximately one-half of the values obtained for cells of actively dividing vegetative cultures growing from a small inoculum (Table 3, Expts. 1 and 2). In Expts. 5 and 6 (Table 3) assays made after incubation for 20 min. indicated that a 65% increase in cellular vitamin B_6 occurred during spore germination, i.e. during the period when the spore lost heat resistance and became permeable to stains but had not increased markedly in size (Powell, 1950). This apparent increase was partly false; it was calculated on a basis of cell dry wt. and should be corrected to account for the 30% decrease in spore dry wt. which occurs during germination (Powell & Strange, 1953). In this connexion, the vitamin B_6 content of spores (0·2 mg. dry wt./ml.) allowed to develop for 1 hr. at 37° in the enriched potato-extract medium was compared with that of spores incubated for 1 hr. in 40 mM-sodium potassium phosphate, pH 7·3, containing 4 mM-L-alanine (Table 4). Germination was complete in the simple amino acid medium (Powell, 1950), but no development into vegetative cells occurred. There was a threefold increase in cellular vitamin B_6 during spore development in the enriched potato-extract medium and only a slight increase in the simple amino acid medium. The slight increase could be entirely accounted for by the change in spore dry wt. during germination.

In a previous study (Powell & Strange, 1957) the DAP decarboxylase activity of cells of *B. sphaericus* from cultures at various stages of growth and sporulation was determined. It was concluded that no DAP decarboxylase synthesis occurred during the development of the spore into a vegetative cell. This conclusion was the result of measurements made on cultures in the enriched potato-extract medium inoculated with 2 mg. dry wt. of spores/ml. The results obtained in the present study suggested that DAP decarboxylase synthesis during spore development might be demonstrable in more dilute suspensions. In Expts. 5 and 6 (Table 3), the DAP

Table 3. *Total vitamin B_6 content of resting, germinated and developing spores of* Bacillus sphaericus *N.C.T.C.* 9602 *in enriched potato-extract medium at* 37°

In Expt. no. 1 values in parentheses refer to pyridoxal phosphate (μm-moles/g. dry wt. of cells). The inocula (mg./dry wt. of spores/ml.) were 1·0 and 0·6 for Expts. 1 and 2 respectively and 0·2 for Expts. 3–6.

Time of incubation (hr.)	Appearance of culture	Vitamin B_6 content (μm-moles/g. dry wt. of cells)					
		Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
0	Resting spores	95 (57)	83	67	68	83	81
0·33	Germinated spores	—	—	—	—	136	136
1·0	Swollen germinated spores	124 (53)	106	200	188	215	219
2·0	Very short granular vegetative cells	148 (60)	124	—	—	—	—
3·0	Short granular vegetative cells	—	—	158	163	—	—
7·0*	Featureless vegetative cells*	426 (250)	510	—	—	—	—

* Separate cultures grown from a small inoculum (0·05–0·10 μg. dry wt. of spores/ml.) for comparison assay.

Table 4. *Vitamin B_6 content and diaminopimelic acid decarboxylase activity of resting and developing spores of* Bacillus sphaericus *N.C.T.C.* 9602

The spore inoculum was 0·2 mg. (dry wt.) of spores/ml.

Medium	Appearance of culture after 1 hr. at 37°	Vitamin B_6 content (μm-moles/g. dry wt. of cells)	DAP decarboxylase activity ($Q^{N_2}_{CO_2}$)
Distilled water	Resting spores	72	1·4
4 mM-L-Alanine in 40 mM-phosphate buffer, pH 7·3	Germinated spores	93	2·0
Enriched potato-extract medium	Short granular vegetative cells	255	4·7

decarboxylase activity of spores (0·2 mg. dry wt./ml.) allowed to develop for 1 hr. in the enriched potato-extract medium was compared with that of resting spores. In Expt. 5, the $Q_{CO_2}^{N_2}$ of resting spores was 3·2 and of developing spores 5·1. In Expt. 6 the corresponding $Q_{CO_2}^{N_2}$ values were 3·2 and 5·4, and in a similar experiment not included in Table 3, 3·6 and 11·5. In the experiment summarized in Table 4 the DAP decarboxylase activity of resting spores was compared with that of spores allowed to develop for 1 hr. in the enriched potato-extract medium and in a buffered solution of L-alanine. The $Q_{CO_2}^{N_2}$ values obtained were 1·4, 4·7 and 2·0. The apparent slight increase in DAP decarboxylase activity during germination in L-alanine could be accounted for by the decrease in spore dry wt. during germination. It should be noted that the DAP decarboxylase assays were made in the presence of an added excess of pyridoxal phosphate.

It seemed possible that first-generation vegetative cells of *B. sphaericus*, having developed under conditions of relatively low vitamin B_6 concentration and DAP decarboxylase activity, might contain DAP in amounts comparable with those in resting spores. In Expts. 2 and 3 (Table 3) resting spores contained 1·5 %, and spores incubated for 1 hr. in the nutrient medium 0·2 % (w/w) of DAP. After a further incubation for 1 hr. in Expt. 2, and for 2 hr. in Expt. 3, no DAP could be detected in 10 mg. dry wt. of cells.

Effect of pyridoxine and its antagonists on growing cultures of Bacillus sphaericus

Pyridoxine, pyridoxal or pyridoxal phosphate was added to the enriched potato-extract medium to give concentrations up to 0·5 mM. Batches of medium (10 ml.) with and without additions were inoculated with *B. sphaericus*, strains N.C.T.C. 9602, 7582, or a laboratory strain of *B. sphaericus* var. *rotans*. The cultures were shaken at 37° for 18 hr. and examined in stained films. The additions had no apparent inhibitory effect on sporulation and there was normal growth. In a similar series of tests, the addition of deoxypyridoxine (2 mM) or isonicotinyl hydrazide (INH) (10 mM) had no stimulatory effect on the sporulation of *B. sphaericus* N.C.T.C. 7582 or its asporogenous variant (Powell & Hunter, 1955). Vegetative cells from 9 hr. cultures growing in the presence of the inhibitors were examined for their DAP content. No DAP was detected chromatographically (Rhuland *et al.* 1955) in hydrolysates containing 5 mg. dry wt. of cells.

DISCUSSION

In previous studies it was shown that DAP was a constituent of sporulating cells and spores of *B. sphaericus* but not of vegetative cells, whereas in other *Bacillus* species vegetative cells, sporulating cells and spores all contained approximately equal amounts of DAP (Powell & Strange, 1956; Strange & Dark, 1956*a*; Powell & Strange, 1957). On a basis of cell dry wt., the DAP content of *B. sphaericus* cells increased approximately twofold as the sporangia were shed and the spores freed (Powell & Strange, 1957). It therefore seemed likely that DAP was a constituent of the spore, but not of the surrounding sporangium. As in other *Bacillus* species, the DAP of *B. sphaericus* spores was identified as a constituent of a soluble hexosamine-containing peptide liberated during spore germination or mechanical disintegration and derived from the spore coat (Strange & Dark, 1956*b*; Powell & Strange, 1957; Strange & Dark, 1957). In contrast with other *Bacillus* species (Antia *et al.* 1957) vegetative cells of *B. sphaericus* possessed a very active DAP decarboxylase system ($Q_{CO_2}^{N_2}$ 20–30) requiring pyridoxal phosphate as a cofactor (Powell & Strange, 1957). It is possible that in *B. sphaericus* *meso*-DAP is produced during vegetative growth but immediately decarboxylated. Alternatively both DAP and the structurally related dipicolinic acid may be synthesized from a common precursor when sporulation begins. It was first thought that the appearance of *meso*-DAP in sporulating cells of *B. sphaericus* might be associated with a decrease in their DAP decarboxylase activity, brought about by a failure in the synthesis of the apoenzyme or its cofactor. It was found, however, that the DAP decarboxylase activity of sporulating cells was still approximately 50 % that of vegetative cells and that their pyridoxal phosphate content was equal to that of vegetative cells. On the other hand, the DAP decarboxylase activity of free spores corresponded to only 10 % and their pyridoxal phosphate to 25 % of that of vegetative and sporulating cells. Free resting spores are metabolically inert and their protoplasmic constitution is very different from that of vegetative cells. It is therefore likely that the protoplasm and the metabolic activity of developing spores are different from that of the surrounding sporangia: thus the DAP decarboxylase activity of developing spores of *B. sphaericus* may be considerably lower than the measured overall activity of sporulating cells and may closely approach that of free spores. The incorporation of DAP rather than lysine into the spore peptide might be accounted for on this basis, if, as again seems likely, the construction of the spore coat depends upon the metabolic processes of the developing spore itself. A preparation of spore peptide isolated from germination exudate of *Bacillus megaterium* (Strange & Powell, 1954) was not decarboxylated by a disintegrated suspension of vegetative cells of *B. sphaericus* (J. F. Powell,

unpublished work). It appeared therefore that once incorporated into the spore peptide, DAP was no longer susceptible to attack by the decarboxylase.

It has been shown (Davison, 1956) that INH inhibited certain enzymes which require pyridoxal by combining with the cofactor. Hoare (1956) showed that lysine and DAP decarboxylases are inhibited by INH (0·5 mM). In the present study, vegetative cells of *B. sphaericus* growing in the presence of a concentration of INH (10 mM) slightly lower than that which caused growth inhibition, still contained no DAP. In this connexion it was also interesting that no DAP could be detected in first-generation vegetative cells of *B. sphaericus* which had developed under conditions of relatively low DAP decarboxylase activity.

It appears that no vitamin B_6 or DAP decarboxylase synthesis occurred during spore germination when this proceeded in the presence of the specific germination stimulant alone. Vitamin B_6 and DAP decarboxylase synthesis did occur in the presence of nutrients as a result of the further development and growth of the germinated spore. This is in accord with previous observations on the different natures of the germination process and the subsequent phases of development and growth, readily demonstrated by their widely different susceptibilities to various inhibitors (Powell, 1950, 1951; Roth & Lively, 1956; Murty & Halvorson, 1957). The increase in vitamin B_6 content and DAP decarboxylase activity during spore development was more marked when the size of the spore inoculum was reduced. In the most dilute suspensions used (0·2 mg. dry wt./ml.) the cell concentration was approximately one-eighth of the maximum cell concentration reached in cultures growing freely from a very small inoculum. The microscopic appearance of first-generation vegetative cells developing in suspensions inoculated with 0·2 mg. dry wt. of spores/ml. and their failure to divide during the 3 hr. period of an experiment suggested that the spore inoculum should have been reduced even further to simulate normal growth conditions.

SUMMARY

1. In sporulating cells of *Bacillus sphaericus* N.C.T.C. 9602, $\alpha\epsilon$-diaminopimelic acid (DAP) was present as the *meso*-isomer.

2. The total vitamin B_6 content of vegetative and sporulating cells of *B. sphaericus* ranged between 300 and 550 μm-moles and the pyridoxal phosphate content between 180 and 330 μm-moles/g. dry wt. of cells. The corresponding values for free resting spores were 70–200 and 50–95 μm-moles/g. dry wt. of cells.

3. In two cultures of a laboratory strain of *Bacillus cereus* the total vitamin B_6 content of vegetative cells was 162 and 224 μm-moles/g. dry wt. of cells and that of free resting spores 99 and 73 μm-moles/g. dry wt. of cells.

4. The vitamin B_6 content and DAP decarboxylase activity of *B. sphaericus* spores increased approximately threefold during development and growth for 1 hr. in a nutrient medium. Although germination occurred there was no increase in cellular vitamin B_6 or DAP decarboxylase activity when spores of *B. sphaericus* were incubated for 1 hr. with L-alanine.

5. Some possible relationships between DAP metabolism and sporulation in *B. sphaericus* are discussed.

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A Corticotrophin-Releasing Factor: Partial Purification and Amino Acid Composition

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Neurohypophysial or hypothalamic tissue, in combination with a sympathetic amine, increases significantly the release of adrenocorticotrophic hormone (ACTH) from rat anterior-pituitary tissue *in vitro* (Saffran & Schally, 1955*a*). A corticotrophin-releasing factor (CRF) was detected in posterior-pituitary extracts and was shown to be distinct from oxytocin, vasopressin and histamine (Saffran, Schally & Benfey, 1955; Schally & Saffran, 1956). The active material has now been concentrated several thousand-fold by serial paper chromatography and the amino acids present in an acid hydrolysate of a concentrate have been identified.

MATERIAL AND METHODS

Tissue preparations

Dilute acetic acid extracts. Acetone-dried powders of neurohypophysial, hypothalamic or brain tissue were extracted with hot aq. 0·5% acetic acid, as described by Stehle (1933).

Precipitation with methanol. To 50 ml. of a 0·5% acetic acid extract of 2·5 g. of an acetone-dried powder of posterior-pituitary tissue was added 200 ml. of methanol. The mixture was kept at −20° overnight and was centrifuged in lots of 5 ml. per tube at 5000 rev./min. in the high-speed head of the International refrigerated centrifuge, model PR-2, temperature 0°. Each lot of the sediment was washed twice with 0·8 ml. of a mixture of 4 parts of methanol and 1 part of 0·5% acetic acid. The sediment was dissolved in 0·5% acetic acid, the solutions were pooled and the volume was adjusted to 12·5 ml. This yellowish, slightly turbid solution was kept in the refrigerator.

Protopituitrin. Protopituitrin is the name given by Parke, Davis and Co. to fraction C in Waring & Landgrebe's (1950) summary of the procedure of Kamm, Aldrich, Grote, Rowe & Bugbee (1928) for the separation of the pressor and oxytocic principles from posterior-pituitary powders. Material of either ox or hog origin was kindly supplied by Parke, Davis and Co., Detroit, Mich., U.S.A., and contained 12–30 i.u. of pressor activity and 11 to 15 i.u. of oxytocic activity/mg. One such preparation contained 0·25 i.u. of corticotrophic activity when assayed by the method of Saffran & Schally (1955*b*). Similar material of porcine origin was also obtained from N.V. Organon, Oss, Holland. One batch of the latter preparation was treated by the supplier with oxycellulose, according to the method of Astwood, Raben & Payne (1952), resulting in a reduction to one-tenth in the content of ACTH.

Brain and hypothalamic extracts. These products, of porcine origin, were supplied by Nordic Biochemicals Ltd., Montreal. An acetone-dried powder of whole brain was treated by the procedure of Kamm *et al.* (1928) for the preparation of Protopituitrin from posterior-pituitary powder to yield a small amount of residue designated as brain Protopituitrin. Acetone-dried powders of separated hypothalami were extracted with acetic acid and the extract was treated with oxycellulose, according to the method of Astwood *et al.* (1952). The unadsorbed fraction and the adsorbed material eluted with 0·1 N-HCl were lyophilized to yield white powders. The hypothalamic material insoluble in acetic acid was extracted with hot aq. 0·5% acetic acid.

Bioassay

Adrenocorticotrophic hormone. The *in vitro* bioassay of Saffran & Schally (1955*b*) was used for the estimation of ACTH in the tissue preparations and in the CRF test outlined below.

Corticotrophin-releasing factor. The detection of CRF activity was carried out by the method of Schally & Saffran (1956), which is a modification of the original method of Saffran & Schally (1955*a*). Anterior-pituitary tissue of the rat was cut into halves, one of which served as the control tissue to obtain the 'unstimulated' release of ACTH, and the other half was incubated for 1 hr. in the presence of the sample to be tested. A positive test for CRF was denoted by a significantly greater release of ACTH from the test tissue, indicated by a lower fiducial limit greater than 1. The potency of the CRF preparations was estimated from the lowest dose that caused a significant stimulation of the release of ACTH. In the earlier tests, 0·2 mM-(−)-noradrenaline was added to keep conditions the same as in the original method (Saffran & Schally, 1955*a*). Arterenol is, however, not required to demonstrate CRF activity in tissue extracts. In more recent tests, the pituitary tissue was pre-incubated for 1 hr. before the addition of CRF to the test tissue. The pre-incubation medium was discarded (cf. Saffran & Bayliss, 1953). Pre-incubation decreased and made more consistent the release of ACTH by the control tissue. In all cases the material under test was added to the experimental tissue at the start of the incubation period and to the control tissue at the end of the incubation period. In this way, all the adrenal tissue in the assay for ACTH was exposed to the materials added with the test sample. This precaution is important in view of the contamination of certain posterior-pituitary preparations by significant amounts of ACTH. In addition, any possible potentiation of the effect of ACTH at the adrenal level is eliminated.

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Colorimetric methods

Peptides. The method of Lowry, Rosebrough, Farr & Randall (1951) was used to estimate peptides.

Ninhydrin. Amino acids and related compounds were determined by the ninhydrin method of Moore & Stein (1954). The colour was developed in a total volume of 2 ml. The reagent blank was minimized by using 2-methoxyethanol redistilled over $FeSO_4$, and recrystallized ninhydrin and hydrindantin.

Paper chromatography

All solvents, except the reagent-grade acetic acid, were redistilled before use. The water was glass-distilled.

For most chromatograms, Whatman no. 3 filter paper (22½ in. × 18¼ in.) was used. The paper used in the final stages of the separation of the active material was washed for several days by successive descending chromatography with 2N-acetic acid, water, ethanol and acetone. The material in 0·5% acetic acid solution was applied as a streak at the starting line.

Butan-1-ol–acetic acid–water (4:1:5, *by vol.*). This system (Partridge, 1948) was used for descending chromatography. The load at the starting line was 2·5–3 mg./cm. and the time of development was 40–48 hr. at 28°. As the developing solvent ran off the paper, the R_F of the zones could not be determined; therefore lysine, which has R_F 0·18 in this system, was run as a reference substance on every chromatogram.

m-*Cresol saturated with water.* This solvent was used for ascending chromatography, with a load of 1·5–2 mg./cm. for 40–48 hr. at 28°. Occasional batches of *m*-cresol inactivated the CRF and could not be used even after redistillation.

Acetone–water (3:2, *v/v*). This system (Bentley & Whitehead, 1950) was used for ascending chromatography at 4°, with a load of 1–1·2 mg./cm. and a development period of 18–20 hr. Equally sharp separation of the zones resulted with or without urea under these conditions; therefore urea was omitted.

Propan-1-ol–water (4:1, *v/v*). Used for ascending chromatography, this system was run with a load of 0·5–1 mg./cm. at 28° for 20–24 hr.

Location of substances on paper. Amino acids and peptide material were detected by spraying narrow strips of the chromatograms with 0·2% ninhydrin in butanol, followed, after development of the ninhydrin colour, by a spray of 1% copper nitrate in ethanol. Vasopressin and oxytocin give a pink colour with this treatment. Ultraviolet-light-absorbing or fluorescing material, such as vasopressin and oxytocin, were located by examination in ultraviolet light. CRF and, in some cases, vasopressin, were located by biological tests on eluates of appropriate zones of the chromatogram.

Elution. Selected zones were cut horizontally from the chromatograms and were eluted by descending chromatography with 0·5% acetic acid for 8–20 hr., depending upon the area of the paper. The eluates, in 100 ml. beakers, were concentrated to small volumes or to dryness *in vacuo* over NaOH.

Amino acid analysis. The amino acids were determined as described by Peart (1956), except that the two-dimensional chromatographic method of Levy & Chung (1953) was used to separate the amino acids. The chromatogram was sprayed with 1% ninhydrin in butan-1-ol containing 8% (v/v) acetic acid because of the alkaline buffer used in the chromatographic system. The ninhydrin colour was developed overnight in darkness. Proline was determined by the method of Chinard (1952) as modified by Bell, Howard, Shephard, Finn & Meisenhelder (1956).

Chemical treatment of corticotrophin-releasing factor

Partial acid hydrolysis. A sample was heated in 2·2N-HCl for 90 min. at 110° in a sealed ampoule.

Total acid hydrolysis. Amounts (0·1–1·0 mg.) were heated with 0·2–2·0 ml. of 6N-HCl for 20 hr. at 110° in a sealed tube.

Reduction. Reduction with thioglycollate was carried out according to Ames, Moore & Van Dyke (1950), except that the concentration of thioglycollic acid was increased to 0·05M and the time of reaction to 24 hr.

Oxidation. Performic acid oxidation was carried out by the method of Sanger (1949) or Popenoe & du Vigneaud (1953). This procedure destroys the pressor and ACTH activities (Dedman, Farmer & Morris, 1955).

RESULTS

Activity of neural-tissue extracts

Several preparations of neural origin were explored for CRF activity in the search for a suitable starting material for the concentration of CRF. Of the preparations examined (Table 1), hypothalamic extracts and Protopituitrin exhibited consistent activity at a dose of 10 μg. Protopituitrin was chosen for further study because it is available in relatively large amounts as a stage in the preparation of vasopressin and oxytocin. The apparent inhibition of release of ACTH by higher doses of some preparations is partly explained by the presence of ACTH in these preparations. The addition of external ACTH to the test system masks the stimulated release of ACTH by the isolated pituitary tissue. Table 2 lists the ACTH content of two posterior-lobe preparations and shows how treatment of Protopituitrin with oxycellulose decreases markedly the contamination by ACTH.

Earlier experiments (Saffran *et al.* 1955) had suggested that CRF had the properties of a peptide. Accordingly paper-chromatographic methods used for the separation of peptides were applied to the concentration of CRF from Protopituitrin. Protopituitrin separated into a large number of ninhydrin-staining components in each of the solvent systems, and the zone containing CRF activity in each system was located by testing eluates of portions of the paper. Both ninhydrin and biological-activity patterns were reproducible. The systems were then applied serially to concentrate the CRF.

Table 1. *Corticotrophin-releasing activity of extracts of neural tissue*

For details of preparations see text.

Preparation	Dose (μg./flask)	No. of tests	No. of tests with lower fiducial limit >1	Mean ratio of ACTH release $\left(\frac{\text{experimental}}{\text{control}}\right)$
Brain extract	1	1*	0	1·2
Brain extract	10	1*	0	0·5
Brain extract	100	1*	0	1·0
Brain extract	1000	1*	0	0·7
Brain Protopituitrin	10	1	0	0·9
Brain Protopituitrin	100	1	0	1·3
Brain Protopituitrin	500	1	0	2·3
Hypothalamic extract, unabsorbed by oxycellulose	10	2	2	2·9
Hypothalamic extract, absorbed by oxycellulose	10	1	1	2·4
Hypothalamic extract, insoluble in acetic acid	10	1	1	2·4
Posterior-pituitary extract, hog	10	4*	1	1·5
Posterior-pituitary extract, hog	14·5	7*	1	1·3
Posterior-pituitary extract, hog	48	3*	2	1·3
Posterior-pituitary extract, hog	72	2*	0	0·9
Methanol precipitate	1	4*	1	1·5
Posterior-pituitary extract, ox	10	4*	1	1·4
Posterior-pituitary extract, ox	100	2*	0	0·9
Protopituitrin	1	4	1	1·3
Protopituitrin	10	11	8	2·2
Protopituitrin	100	2	0	1·4
Protopituitrin, oxycellulose-treated	1	1	1	1·8
Protopituitrin, oxycellulose-treated	10	1	1	2·6

* (±)-Noradrenaline 0·4 mM. The remainder of the tests were with pre-incubated tissue.

Table 2. *Content of adrenocorticotrophic hormone in posterior-pituitary preparations*

Figures in parentheses are the 95% confidence limits.

Preparation	ACTH (i.u./mg.)
Posterior-pituitary extract, ox	0·19 (0·09–0·44)
Protopituitrin	0·25 (0·11–0·54)
Protopituitrin, oxycellulose-treated	0·03 (0·025–0·035)

Stages of purification

(1) *Chromatography in butanol–acetic acid–water.* The CRF activity was recovered in the zone with R_F 0·05–0·2 (lysine = 0·18). This zone contained one-half to one-quarter of the weight of the Protopituitrin applied to the starting line, so that the active material eluted from this zone was concentrated two to four times.

(2) *Chromatography in* m-*cresol.* The active material from step 1 was chromatographed in the *m*-cresol system. After development, the paper was dried overnight in the fume hood at room temperature, with an extra fan to increase the circulation of air within the hood. The area with R_F 0·46–0·64 consistently contained the activity. Eluates of this zone were taken to dryness several times *in vacuo* over NaOH to remove traces of *m*-cresol. The material contained one-twenty-fifth to one-fortieth of the weight of Protopituitrin.

(3) *Rechromatography in* m-*cresol.* The eluate from step 2 was rechromatographed on washed filter paper in the *m*-cresol system to separate completely from CRF the faster-running peptides, including vasopressin (R_F 0·85), which tended to tail at the relatively high loads used in step 2, and thus contaminate the CRF. The zone with R_F 0·46–0·64 again contained the activity in about $\frac{1}{170}$ of the starting weight of Protopituitrin. Washed paper was used in this and in the subsequent two steps to minimize contamination of the preparation with substances such as metals which might hasten inactivation.

(4) *Chromatography in acetone–water.* The material concentrated in step 3 was chromatographed in the actone–water system, in which the activity was located in the zone with R_F 0·35–0·46. This zone contained $\frac{1}{2200}$–$\frac{1}{2600}$ of the weight of Protopituitrin. The weight was estimated by the method of Lowry *et al.* (1951) and by weighing, when larger-scale separations were run.

(5) *Chromatography in propanol–water.* The material from step 4 was chromatographed as two spots in the propanol–water system. At this stage of purification the ninhydrin-staining spots coincided with ultraviolet-light-absorbing or fluorescing areas. Examination in ultraviolet light revealed two faintly fluorescing spots with R_F 0·07–0·22 and 0·33–0·43, and a strongly absorbing spot with R_F 0·22–0·33. Activity was consistently located in the fluorescing zones. The absorbing zone was not active in limited tests at low-dose levels. The zone with R_F 0·07–0·22 contained $\frac{1}{4600}$–$\frac{1}{5000}$ of the

weight of Protopituitrin, as estimated by the method of Lowry *et al.* (1951). The other active zone contained even less material.

The steps in the purification of CRF are outlined in Table 3. At each step, a portion corresponding to 10 μg. of Protopituitrin was used in the biological test for CRF (Table 4). This dose is consistently active, suggesting that there were no serious losses in activity during the course of purification. In contrast, similar doses of material eluted from other zones in the first four steps were inactive.

The facilities of our Laboratory limited the capacity of each run to 3 g. of Protopituitrin. Table 3 illustrates the enormous amount of labour involved in the spotting, running and elution of approximately 50 chromatograms needed to concentrate 600 μg. of CRF.

Table 5 lists the R_F of CRF, the vasopressins, oxytocin and α-corticotrophin in the four solvent systems to demonstrate the ability of the combination of these systems to separate CRF from the other known hormones in the starting material.

Amino acid composition

Table 6 lists the amino acids found in acid hydrolysates of two preparations of CRF with R_F 0·07–0·22 in the propanol–water system, one made from Protopituitrin, provided by Parke, Davis and Co., and the other from similar material, treated with oxycellulose, supplied by N. V. Organon. The two preparations differ significantly in their content of glycine, tyrosine and alanine. Both consistently stimulated the release of ACTH at a dose of 0·5 μmg., but the preparation made from Protopituitrin was decidedly more potent. Traces of threonine and of an unknown ninhydrin-reacting material (R_F 0·6 in butanol–acetic acid–water and R_F 0·18 in phenol–*m*-cresol, pH 9·3) were found in the preparation made from Organon starting material.

Chemical treatment

The biological activity in one of two preparations was abolished by reduction with thioglycollic acid, oxidation with performic acid and extensive acid hydrolysis (Table 7). However, treatment with

Table 3. *Steps in the concentration of corticotrophin-releasing factor*

Solvent system	Wt. applied (mg.)	No. of chromatograms	Average concentration factor
Butanol–acetic acid–water	3000	30	1
m-Cresol	900–1800	10–20	3
m-Cresol rechromatography	90–140	2–3	33
Acetone–water	20	1	170
Propanol–water	1·2–1·5	1	2400
Final product	0·6	44–55	4800

Table 4. *Typical release of adrenocorticotrophic hormone by corticotrophin-releasing factor at various stages of preparation*

Stage	Dose (μmg.)	% of control	95% fiducial limits
m-Cresol, rechromatographed	60	630	330–1200
m-Cresol, rechromatographed	6	350	190–670
Acetone–water	4	380	150–960
Propanol–water	2	300	150–600
Propanol–water	1·1	270	190–390
Propanol–water	0·9	400	240–690

Table 5. *R_F values of oxytocin, vasopressin, adrenocorticotrophic hormone and corticotrophin-releasing factor*

	Butanol–acetic acid–water	R_F in *m*-cresol–water	Acetone–water	Propanol–water
Oxytocin	0·37	0·85	0·71	0·22
Arginine vasopressin	0·16	0·85	0·65	0·18
Lysine vasopressin	—	0·85	0·69	—
α-Corticotrophin*	0	1·0	—	—
CRF (zone)	0·05–0·20	0·46–0·64	0·35–0·46	0·07–0·22 0·33–0·43

* Personal communication from Professor C. H. Li.

2·2 N-HCl at 110° for 90 min. does not apparently affect the activity of a CRF preparation at the third step of the purification procedure.

DISCUSSION

At present, the greatest weight of evidence supports the hypothesis that the release of ACTH from the adenohypophysis is governed by a material elaborated in the hypothalamic area of the brain (Harris, 1951). Proof of this hypothesis requires the isolation of material, from the hypothalamus, which activates the release of ACTH from the pituitary in a system in which the response to stress has been abolished by some means. The existence of a special, short vascular route (the hypophysial-portal system) for this material suggests that its biological activity is relatively short-lived. The anatomical relationship between the hypothalamus and the neurohypophysis further implies that the substance may be found in the posterior lobe of the pituitary body as well (Zuckerman, 1952), in a manner analogous to the hormones of the posterior lobe, oxytocin and vasopressin (Scharrer & Scharrer, 1954). As a rule, the hormones elaborated by any one endocrine organ are members of the same chemical family. If the hypothalamus–neurohypophysis system obeys this rule, then the hypothetical substance suggested by Harris (1951) and others should be chemically similar to the peptide hormones, vasopressin and oxytocin. However, lipid, lipoprotein and protein, as well as peptide preparations, have been claimed to have the properties of the hypothetical substance.

Slusher & Roberts (1954) reported the concentration of a lipoprotein and a lipid from cow posterior hypothalamus that caused eosinopenia and depletion of adrenal ascorbic acid in intact rats. Tests in normal animals are of dubious value. Roberts (1955) added that these fractions also showed activity in rats rendered unresponsive to stress by lesions in the hypothalamus, but a paper describing this work has not yet appeared.

Recently, Porter & Rumsfeld (1956) reported that plasma-protein fractions prepared from 'hypophyseal portal-vessel blood' caused significant falls in the adrenal ascorbic acid of intact rats treated with hydrocortisone, whereas similar fractions from systemic blood were inactive. Because only about 0·5 μmg. of our peptide material is active in our test, perhaps these lipoprotein and protein fractions, which were administered in relatively large doses, contained some adsorbed CRF, accounting for their activity.

Other authors (e.g. Martini & Morpurgo, 1955) suggested that oxytocin and vasopressin themselves were able to stimulate the release of ACTH and other hormones from the adenohypophysis. However, evidence from our Laboratory (Saffran *et al.* 1955; Schally & Saffran, 1956) and from Guillemin's laboratory (Guillemin & Rosenberg, 1955; Guillemin, Hearn, Cheek & Housholder, 1957) pointed to the presence in commercial preparations of vasopressin of another peptide component which stimulated the release of ACTH by adenohypophysial tissue *in vitro*. This paper shows the active material to be chemically related to the other hormones of the hypothalamus–neurohypophysis system, vasopressin and oxytocin.

Table 6. *Molar proportions of amino acids found after total acid hydrolysis of corticotrophin-releasing factor*

Amino acid	CRF prepared from starting material supplied by: Parke, Davis and Co.	Organon
Cystine	0·89	1·06
Aspartic acid	0·89	1·27
Glutamic acid	0·89	1·18
Lysine	0·54	0·96
Phenylalanine	0·83	0·90
Proline	1·10	1·26
Tyrosine	1·00	0
Glycine	1·00	2·00
Serine*	0·98	1·11
Histidine	0·64	1·10
Alanine	0·22	0·73
Arginine	0·33	0·36

* Corrected for 10% destruction, cf. Levy, Geshwind & Li (1955).

Table 7. *Chemical treatment and activity of corticotrophin-releasing factor*

Preparation	Treatment	Dose (μg.)	Release of ACTH (% of control)	95% limits (%)
80% Methanol precipitate	0·1 M-Sodium phosphate buffer, pH 7·4 (control)	1	290	160–520
	Thioglycollate in phosphate buffer	1	98	50–190
	Formic acid (control)	5	245	130–464
	Performic acid	1	86	54–137
	Performic acid	5	90	68–120
	Performic acid	5	142	62–322
m-Cresol, rechromatographed (Table 3, step 3)	2·2 N-HCl, 90 min.	0·33	320	210–480
	6 N-HCl, 22 hr.	0·33	110	80–140

That CRF is distinct from oxytocin and vasopressin was first shown in early experiments in which the activity was separated from vasopressin by paper chromatography in one system (Saffran *et al.* 1955). The present work utilizes the high resolving power of four successive chromatographic systems to isolate CRF. As depicted in Table 4, chromatography in butanol–acetic acid separates CRF (R_F 0·05–0·2) from oxytocin (R_F 0·37) as well as from a large number of small peptides (high R_F) and of very basic peptides ($R_F \leqslant 0{\cdot}05$). This system was used previously by Benfey (1953) for the separation of vasopressin and oxytocin. The *m*-cresol system separates CRF (R_F 0·46–0·64) from ox and hog vasopressins (R_F 0·85) and from α-corticotrophin (R_F approx. 1·0). A further separation of CRF from these hormones is brought about by the acetone–water step. This separation, as well as the chemical analysis, ensures that the final product is neither vasopressin nor oxytocin. The final, propanol–water, step resolves the active material into two or more equally-potent spots, whose relationship will be the subject of further investigation. The sequence in which the solvent systems are used is critical, because the last two systems are more sensitive to overloading and to the presence of gummy contaminants in Protopituitrin. The final product is relatively unstable, but insufficient quantities were available to attempt systematically to stabilize it, although precautions were taken to avoid contamination with heavy metals or contact with peroxides in the solvents.

The material prepared by Guillemin *et al.* (1957) from hypothalamic and neurohypophysial extracts, and called by them fraction D, resembles in its properties the CRF prepared in our Laboratory. Fraction D is chromatographically distinct from vasopressin, promotes the release of ACTH from isolated anterior-lobe tissue, is most unstable in the purified form (fraction ΔD), and is apparently a small peptide. According to Guillemin *et al.* (1957) fraction D may also be obtained, although by more drastic extraction procedures, from extra-neural sources as well. Fraction D exerts activity at doses ranging upward from 0·5 μg., whereas our most highly purified material has detectable activity, when freshly prepared, at 1/1000 of that dose. Probably variations in assay procedures, as well as a different degree of purification, contribute to the difference in the minimal effective dose.

Lysine vasopressin and CRF have seven or eight amino acids in common; in addition, the CRF preparations consistently contain major amounts of serine and histidine, and alanine and arginine are found in smaller, variable proportions. This indicates that the CRF preparation is not yet chemically pure, although it is possible that a family of similarly active peptides may exist. The composition and specificity of CRF from various species will have to be studied in view of the differences between hog and ox vasopressin (Van Dyke, Adamsons & Engel, 1955).

Guillemin *et al.* (1957) have discussed the physiological significance of CRF, whose biological activity has thus far been demonstrated in a test *in vitro* on isolated tissues.

SUMMARY

1. Neurohypophysial and hypothalamic extracts contain a corticotrophin-releasing factor (CRF) that stimulates the release of adrenocorticotrophic hormone (ACTH) from isolated anterior-pituitary tissue.

2. Serial paper chromatography in four different solvent systems has been applied to the concentration of the CRF in Protopituitrin, a posterior-pituitary concentrate prepared as a stage in the purification of oxytocin and vasopressin.

3. Purified CRF of neurohypophysial origin significantly increases the release of ACTH at doses as low as 1 μmg.

4. Purified CRF has the chemical and chromatographic properties of a peptide, distinct from oxytocin and vasopressin.

5. After acid hydrolysis of purified CRF, the following amino acids were detected: cystine, aspartic acid, glutamic acid, glycine, proline, lysine, phenylalanine, alanine, serine and histidine.

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The Component Acids and Glycerides of the Depot Fat of an Indian Panther (*Panthera pardus fusca*)

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Little is known about the fatty acid composition and glyceride structure of depot fats of land animals, particularly of those of the Carnivora, and quantitative data for the panther (*Panthera pardus fusca*) fat are not available. A study was therefore undertaken of the fatty acid composition and the nature of the glycerides of the depot fat of a panther of Indian origin.

EXPERIMENTAL

A light-cream-coloured fat was obtained from the abdominal depots of a freshly killed male panther from the forest of Indore (Central India). The saponification equivalent (S.E.) of the fat was 285·6 and iodine value (I.V.) was 62·4 with 3·2% of free fatty acid (as oleic). It was purified by washing an ethereal solution with dilute aqueous alkali, when the S.E. and I.V. of the neutral fat were 284·8 and 62·8 respectively.

Determination of component acids. A sample (140 g.) of the fat was saponified and the mixed fatty acids (130 g.; I.V. 65·5) were recovered. The latter were then resolved into two groups (1 and 2) of simpler mixtures of fatty acids by the lead salt–ethanol method (Hilditch, 1956). Each group of fatty acids was separately converted into the methyl esters. The esters were then subjected to fractional distillation under high vacuum (0·1 mm.) through an efficient electrically heated and packed column (Longenecker, 1937). The composition of each subfraction was calculated from their respective saponification equivalents and iodine values according to the method recommended by Hilditch (1956). The final composition of the fat was then computed from these figures.

Determination of component glycerides. A portion (195 g.) of the neutral fat (I.V. 62·7) was dissolved in acetone (1·95 l.) and the solution kept at 0°, when 20·6 g. (I.V. 40·8) of glycerides crystallized out. The soluble portion, 174·4 g. (I.V. 63·5), was again crystallized from acetone (1·74 l.) at −12°. The soluble portion (93 g.; I.V. 65·7) and the insoluble portion (81·4 g.; I.V. 59·4) were further crystallized separately from acetone (20 g./100 ml.) at −12°; the former gave an insoluble portion (11 g.) with I.V. 60·3 and a soluble portion (82 g.) with I.V. 66·4. The latter gave an insoluble portion (35·5 g.) with I.V. 51·4 and a soluble portion (45·9 g.) with I.V. 62·6. Attempts were made to crystallize all the above fractions from ether at various temperatures, but were unsuccessful.

Different fractions thus obtained were combined according to similarity of iodine values. The procedure gave three fractions of 56·1 g. (I.V. 47·5), 56·9 g. (I.V. 62·1) and 82·0 g. (I.V. 66·4) respectively which were named *A*, *B* and *C*. The mixed acids obtained by the usual method from these fractions were further subjected to the lead salt–ethanol method of separation. These acid fractions were called *AA*, *AB*, *BA*, *BB*, *CA* and *CB* respectively. Each fraction was converted into the methyl ester and fractionated by distillation. Lastly, the possible component glycerides were calculated from the composition of the individual group in the manner described by Hilditch & Meara (1942).

A separate determination of the fully saturated glycerides by the method of Hilditch & Lea (1927) was done to check the computed values.

RESULTS

Table 1 gives data relating to the fractional crystallization of the mixed fatty acids of panther fat by the lead salt–ethanol method.

The component acids of the panther fat are recorded in Table 2.

Table 3 summarizes the results obtained by crystallization of neutral fat from acetone at various temperatures.

Table 4 presents the separation of the mixed acids of glyceride fractions *A*, *B* and *C* by the lead salt–ethanol method.

The component acids of glyceride fractions *A*, *B* and *C* are given [% (mol.)] in Table 5.

The possible component glycerides of the panther fat are given in Table 6, along with the negative result of fully saturated glyceride content in the fat.

Table 1. *Separation of the acids of panther fat by the lead salt–ethanol method*

Group	Solubility of lead salt in ethanol at 15°	Weight (g.)	Weight (%)	Iodine value
1	Insoluble	40·0	33·9	6·6
2	Soluble	78·0	66·1	89·2

DISCUSSION

Fatty acid composition. The panther feeds generally on wild animals such as deer and sheep and occasionally on calves, cows and buffaloes. Only a few of the carnivorous-animal fats have been analysed so far; some are given in Table 7 for comparison with the present work, which shows that panther fat falls into line with other carnivorous-animal fats.

The compositions of the fats in Table 7 are broadly similar. Palmitic and stearic acids are major components among the saturated acids. The present panther fat resembles lion fat in having a lower content of stearic acid than tiger fat, but the arachidic acid is a minor component in all the cases cited above.

In general these fats appear to have been synthesized from the fats in the animals' diet. Lion fat (Hilditch, Sime & Maddison, 1942), tiger fat (Pathak & Agarwal, 1952) and panther fat are very similar in composition and appear to have been synthesized mainly from the depot fat of herbivorous ox (Hilditch & Longenecker, 1937) or sheep (Hilditch & Srivastava, 1949) (Table 7). This is an example of the fact that the composition of depot fat depends on the diet of the animal. Among the

Table 2. *Component acids in groups* 1 *and* 2 *from the whole fat of the panther*

Figures in parentheses indicate unsaturation, e.g. −2·0H means monoethenoid. The figures for component acids are derived from the quantities of fractions actually obtained on fractionation; loss was little and ignored. Hence the figures total 100.

Acids	Component acids of groups (%) 1	2	Total (%)	Fatty acids in the whole fat, excluding non-saponifiables % (w/w)	% (mol.)
Saturated					
Myristic	1·4	0·9	2·3	2·3	2·8
Palmitic	18·1	1·9	20·0	20·1	21·4
Stearic	10·8	2·9	13·7	13·7	13·2
Arachidic	1·1	0·6	1·7	1·7	1·5
Unsaturated					
C_{14}	—	1·8 (−2·0H)	1·8 (−2·0H)	1·8	2·2
C_{16}	0·5 (−2·0H)	10·3 (−2·0H)	10·8 (−2·0H)	10·8	11·6
C_{18}	1·6 (−2·0H)	39·7 (−2·1H)	41·3 (−2·1H)	41·3	40·0
C_{20}	0·4 (−2·0H)	7·9 (−3·1H)	8·3 (−3·1H)	8·3	7·3
Unsaponifiable	0·04	0·06	0·1	—	—

Table 3. *Crystallization of neutral panther fat from acetone*

The figures in parentheses represent ml. of acetone/g. of fat.

Fraction	Description	Weight (g.)	Weight (%)	Iodine value
A	Material insoluble in acetone (10 ×) at 0° and −12°	56·1	28·8	47·5
B	Material soluble in acetone (10 ×) at −12° together with insoluble in acetone (5 ×) at −12°	56·9	29·2	62·1
C	Material soluble in acetone (5 ×) at −12°	82·0	42·0	66·4

Table 4. *Separation of the mixed acids of glyceride fractions A, B and C*

For preparation of fractions see text.

	Solubility of lead salt in ethanol at 15°	Weight (g.)	Weight (%)	Iodine value
Fraction *A*				
AA	Insoluble	20·6	41·6	4·7
AB	Soluble	28·9	58·4	78·2
Fraction *B*				
BA	Insoluble	13·4	26·3	4·9
BB	Soluble	37·6	73·7	79·3
Fraction *C*				
CA	Insoluble	14·0	20·0	5·6
CB	Soluble	56·0	80·0	84·6

Table 5. *Component acids of glyceride fractions A, B and C*

Component acids	*A*	*B*	*C*	Total
Myristic	0·6	0·7	0·7	2·0
Palmitic	8·8	6·1	6·5	21·4
Stearic	5·2	4·3	6·0	15·5
Arachidic	Trace	Trace	0·2	0·2
Tetradecenoic	0·3	0·5	0·7	1·5
Hexadecenoic	2·7	4·8	4·8	12·3
Octadecenoic	10·3	11·9	21·2	43·4
Eicosenoic	1·0	1·0	1·7	3·7

Table 6. *Component glycerides of panther fat*

Calculations in this table are based on the suggestions of Hilditch & Meara (1942).

	A	*B*	*C*	Whole fat
Component acid groups (% mol.)				
Myristic and palmitic	9·4	6·9	7·1	23·4
Stearic and arachidic	5·2	4·3	6·2	15·7
Tetra- and hexa-decenoic	3·0	5·3	5·5	13·8
Octadec- and eicos-enoic	11·3	12·9	22·9	47·1
Component glyceride groups (% mol.)				
(*a*) (i) Disaturated–mono-unsaturated	14·9	4·2	—	19·1
(ii) Monosaturated–diunsaturated	14·0	25·1	40·0	79·1
(iii) Triunsaturated	—	—	1·8	1·8
(*b*) (i) Dipalmito–mono-others	8·3	—	—	8·3
(ii) Monopalmito–di-others	11·5	16·4	21·1	49·0
(iii) Tri-others	9·0	13·0	20·7	42·7
(*c*) (i) Monostearo–di-others	15·7	13·0	18·5	47·2
(ii) Tri-others	13·2	16·4	23·2	52·8
(*d*) (i) Dioleo–mono-others	4·9	9·3	27·1	41·3
(ii) Mono-oleo–di-others	24·0	20·0	14·7	58·7
(*e*) (i) Hexadeceno–di-others	9·0	15·8	16·5	41·3
(ii) Tri-others	19·9	13·5	25·3	58·7
Possible component glycerides (% mol.)				
Mono-unsaturated–disaturated (19·2%)				
Dipalmito-olein	8·3	—	—	8·3
Palmitostearo-olein	6·7	4·2	—	10·9
Diunsaturated–monosaturated (79·0%)				
Dioleopalmitin	4·9	9·3	6·7	20·9
Dioleostearin	—	—	18·5	18·5
Mono-oleohexadecanostearin	9·1	8·8	—	17·9
Mono-oleohexadecenopalmitin	—	7·0	14·7	21·7
Triunsaturated (1·8%)				
Dioleohexadenoic	—	—	1·8	1·8

Table 7. *Component acids (% by wt.) of the fats of some carnivorous animals and of English ox and Indian sheep*

Acids	Lion [Hilditch, Sime & Maddison (1942)]	Tiger [Pathak & Agarwal (1952)]	Panther (Present work)	Ox [Hilditch & Longenecker (1937)]	Sheep [Hilditch & Srivastava (1949)]	Puma [Giral (1945)]
Decanoic	1·4	—	—	—	—	—
Lauric	1·1	—	—	0·2	—	—
Myristic	4·9	1·0	2·3	3·1	2·9	1·3
Palmitic	28·9	22·4	20·1	24·9	27·8	22·4
Stearic	17·8	24·6	13·7	24·1	27·7	26·9
Arachidic	0·1	1·2	1·7	0·8	1·5	3·7
Tetradecenoic	0·6	0·6	1·8	0·4	0·4	—
Hexadecenoic	1·9	7·1	10·8	2·4	2·7	12·6
Oleic	40·3	39·0	39·2	41·8	33·0	26·2
Octadecadienoic	—	4·1	2·1	1·8	3·4	2·3
C_{20-22} unsaturated	3·0	—	8·3	0·5	0·6	4·6

Table 8. *Distribution of acyl groups in the glyceride of panther fat*

	Actual	Computed: Even [Hilditch (1956), pp. 327–332]	Computed: Random [Kartha (1949, 1953)]
Fully saturated glyceride	0	0	6·0
Disaturated–mono-unsaturated	19·1	17·4	28·0
Monosaturated–diunsaturated	79·0	82·6	43·5
Triunsaturated	1·8	0	22·5

unsaturated components tetra- and hexa-decenoic acids are present in lion, tiger and panther fat as well as in ox and sheep fat, though in the lion, ox and sheep fat they are only minor constituents, whereas in tiger and panther fat they occur in appreciable quantities (7·1 and 10·8 %, by wt., respectively). The relatively high content of hexadecenoic acid in tiger and panther fat is noteworthy, and suggests that unsaturation increases from lion to tiger fat and tiger to panther fat. In this respect tiger and panther fat resembles the puma body fat studied by Giral (1945) (Table 7).

Component glycerides. Panther fat contains 19·2 % of mono-unsaturated–disaturated glycerides (dipalmito-olein 8·3 %, palmitostearo-olein 10·9 %), 79·0 % of diunsaturated–monosaturated glycerides (dioleopalmitin 20·9 %, dioleostearin 18·5 %, mono-oleohexadecenopalmitin 21·7 %, mono-oleohexadecenostearin 17·9 %) and 1·8 % of triunsaturated glycerides (hexadecenodiolein 1·8 %).

The distribution of acyl groups in the glycerides of panther fat follows the rule of 'even' distribution, according to which the component fatty acids tend to be distributed as widely as possible among all the triglyceride molecules. The actual values shown in Table 8 are nearly identical with the values computed on the assumption that this rule is valid. In this respect panther fat does not align itself with the general land-animal type.

According to the 'rule of even (widest) distribution' of Hilditch (1956), which appears to hold good for vegetable oils, fully saturated glycerides do not occur in significant proportions unless the unsaturated acid content is below that (about 35 %) necessary to permit of each mixed triglyceride molecule containing one unsaturated acyl group. On this assumption panther fat, which contains about 61 % of unsaturated acids, should not contain any trisaturated glycerides. Land-animal fats in general do not follow this rule, but probably conform to the 'random-distribution' pattern advanced by earlier workers and supported by Kartha (1949, 1953), according to which the fatty acids distribute themselves indiscriminately throughout the glyceride molecules. This pattern, however, has not been found with panther fat.

SUMMARY

1. The composition of the depot fat from a panther (*Panthera pardus fusca*) has been studied. The lead salt–ethanol method was adopted for preliminary separation of the mixed fatty acids into groups differing in unsaturation. The compositions of the resulting fractions were studied by the ester-fractionation procedure.

2. The panther fat aligns itself with other carnivorous-animal fats in its fatty acid composition with some differences in the proportion of saturated and unsaturated acids.

3. The glycerides have been fractionated by crystallization of the neutral fat from acetone. The

composition of each of the three fractions thus obtained has been studied separately and the final possible glyceride composition computed from the resulting data.

4. The glyceride composition of panther fat, unlike that of the fat of certain other land animals, closely follows Hilditch's (1956) rule of 'even' distribution.

The authors wish to express their cordial thanks to the authorities of the Banaras Hindu University for the research facilities provided.

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5-Formylfuroic Acid and the Carbazole Reaction for Uronic Acids and Acidic Polysaccharides

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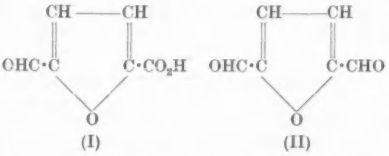
It has been shown (Bowness, 1957) that furfuraldehyde and 5-hydroxymethylfurfuraldehyde in sulphuric acid do not give any substantial coloration with carbazole under the conditions of the procedure (Dische, 1947) for estimating uronic acids.



(I) (II)

In the present work it was found that two other derivatives of furan, 5-formylfuroic acid (I) and 2:5-diformylfuran (II), both give intense colorations under these conditions, and also under the conditions of the modified carbazole reaction which Dische (1950) showed would distinguish between various polyuronides. It appears probable that formylfuroic acid is the principal agent responsible for the formation of colour from uronic acids in these two procedures.

MATERIALS AND METHODS

Materials

5-Formylfuroic acid (m.p. 195°) and 2:5-diformylfuran (m.p. 107°) were prepared and purified by the methods of Cooper & Nuttall (1912). Methyl β-D-glucofuranuronolactone (m.p. 135°) and 1:2-*O*-*iso*propylidene-α-D-glucofuranuronolactone (m.p. 116°) were prepared by the methods of Owen, Peat & Jones (1941). The sulphuric acid for the reaction at 60° (Dische, 1950) was obtained by adding 6 vol. of H_2SO_4 (A.R., Hopkin and Williams Ltd.) cooled to 0°, to 1 vol. of water in an ice bath. Heparin (Pularin) was obtained from Evans Medical Supplies Ltd. The sources or methods of preparation of all other reagents have been described previously (Bowness, 1957).

Methods

Reaction at 100°. Details of this procedure have been described in a previous paper (Bowness, 1957).

The absorption spectra recorded in Fig. 1 and the absorption values in Table 1 were obtained with a Hilger Uvispek (H. 700) spectrophotometer. Measurements were made 2·0–2·75 hr. after removing the sulphuric acid solutions from the boiling water. The solutions without carbazole were read against a sulphuric acid blank; those with carbazole were read against the blank containing carbazole.

Reaction at 60°. The procedure of Dische (1950) was employed, except that 0·2 ml. of a 0·3% (instead of a 0·1%) (w/v) ethanolic solution of carbazole was used. Absorption spectra (Fig. 2 and Table 2) were obtained as described above for the reaction at 100°. Measurements were made 0·5–1·25 hr. after removal of the solutions from the water bath.

RESULTS

Reaction at 100°

In the presence of carbazole. Table 1 shows that furfuraldehyde and 5-hydroxymethylfurfuraldehyde do not give appreciable colours in the reaction

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with carbazole at 100° in sulphuric acid (cf. Bowness, 1957). Galacturonic acid, glucuronolactone, all derivatives and compounds of glucuronic acid tested in this work (not shown in Table 1, but listed in Table 2) and 5-formylfuroic acid gave a substantial coloration with a principal absorption maximum at, or near, 525 mμ and a subsidiary maximum at 395 mμ. 2:5-Diformylfuran also gave a strong colour with carbazole, but the absorption maxima, at 550 and 430 mμ, were quite distinct from those given by formylfuroic acid and the uronic acids. The absorption spectra in the visual region given in the test by glucuronolactone (Fig. 1), galacturonic acid and formylfuroic acid were all of the same shape, though the molar intensities of absorption for the complex with formylfuroic acid were considerably higher than for the other two.

Reaction in the absence of carbazole. The absorption curve of formylfuroic acid in sulphuric acid without carbazole differed slightly from that for glucuronolactone (Fig. 1) and from those for other uronic acid derivatives, but the principal absorption band shown by all these compounds was in the region 298–308 mμ and there was no subsidiary band near 224 mμ as there was for 2:5-diformylfuran.

Glucosaccharic acid heated with concentrated sulphuric acid slowly forms furan-2:5-dicarboxylic acid (Tollens & Yoder, 1901). This last compound was almost certainly responsible for the bottom curve in Fig. 1 ($\lambda_{max.}$ 272 mμ) obtained with calcium saccharate. The absorption intensity increased considerably when the time of heating was prolonged. On mixing with 3 vol. of water the absorption maximum of this solution approached that observed by Hausser, Kuhn, Smakula & Deutsch (1935). No coloration was obtained when carbazole was added to this solution, even after standing for 24 hr.

A solution of 5 mg. of glucuronolactone in sulphuric acid, after treatment under the standard conditions of the reaction at 100°, was mixed with 8 vol. of water. Three-quarters of the acid was neutralized with sodium carbonate and the mixture was extracted twice with 50 ml. of diethyl ether. The ether extract was dried over anhydrous sodium sulphate. The absorption spectrum of the ether extract was very similar to that obtained with a solution of 5-formylfuroic acid in diethyl ether. The positions of the absorption maxima were identical (275 mμ), but the positions of the absorption minima, below 300 mμ, and the ratios of maximum to minimum were somewhat different. The recovery of 5-formylfuroic acid in the ether extract (as estimated by the absorption intensities at $\lambda_{max.}$) was only about 10% of that in the usual procedure for the reaction at 100°.

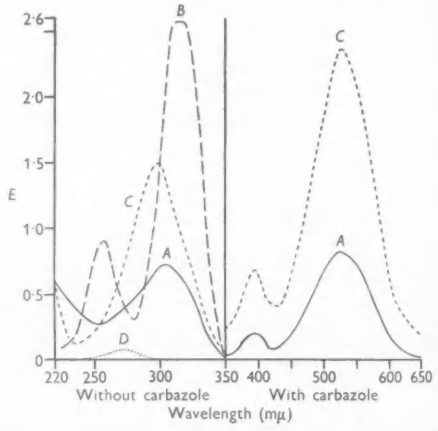

Fig. 1. Absorption spectra of solutions in sulphuric acid, under the conditions of the carbazole reaction at 100°, with (350–650 mμ) and without (220–350 mμ) added ethanolic carbazole. Each solution contained 6 ml. of sulphuric acid and 1 ml. of an aqueous solution of one of the substances listed below; the added carbazole was 0·2 ml. of a 0·2% ethanolic solution. *A*, Glucuronolactone (100 μg.); *B*, furfuraldehyde (100 μg.); *C*, 5-formylfuroic acid (100 μg.); *D*, calcium D-saccharate (120 μg.).

Table 1. *Intensities of absorption of light, and wavelength maxima, of substances in sulphuric acid in the reaction at 100° with and without carbazole*

Procedural details are described under Methods; 0·715 μmole of each substance was tested. Figures in parentheses refer to secondary absorption maxima.

	Without carbazole, in the range 220–350 mμ		With carbazole, in the range 360–650 mμ	
Substance	$\lambda_{max.}$	$E_{max.}$	$\lambda_{max.}$	$E_{max.}$
5-Formylfuroic acid	298	1·494	525 (395)	2·366 (0·687)
2:5-Diformylfuran	310 (224)	1·489 (0·804)	550 (430)	2·278 (1·122)
Furfuraldehyde	317 (257)	1·826 (0·609)	410 (525)	0·071 (0·022)
5-Hydroxymethylfurfuraldehyde	323 (258)	1·472 (0·324)	545 (400)	0·090 (0·067)
Glucuronolactone	302	0·881	525 (395)	1·037 (0·258)
Galacturonic acid	308	0·687	525 (395)	0·715 (0·211)

Reaction at 60°. The absorption curves (Fig. 2) of formylfuroic acid heated in sulphuric acid at 60° (Dische, 1950) were almost exactly the same shape as the corresponding curves for galacturonic acid and the glucuronic acid-containing polysaccharides heparin and oxidized cellulose. The corresponding curves for 2:5-diformylfuran were considerably different. Glucuronolactone, glucuronic acid and most of the derivatives and compounds of this acid (Table 2) showed little or no absorption, either in the ultraviolet region without added carbazole, or in the visible region with carbazole. As in the reaction at 100°, furfuraldehyde and 5-hydroxymethylfurfuraldehyde showed little colour formation with carbazole; the absorption maxima in the ultraviolet, without added carbazole, were different from those for formylfuroic acid and the uronic acids.

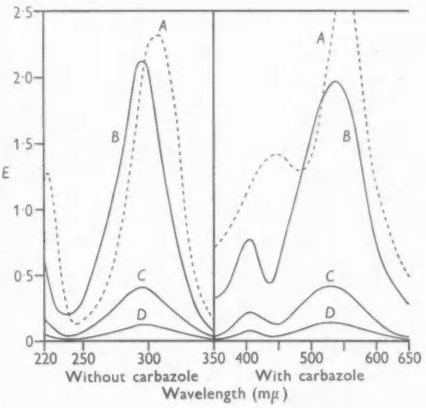

Fig. 2. Absorption spectra of solutions in sulphuric acid, under the conditions of the carbazole reaction at 60°, with (350–650 mμ) and without (220–350 mμ) added ethanolic carbazole. Each solution contained 5·4 ml. of sulphuric acid and 0·4 ml. of an aqueous solution of one of the substances listed below; the added carbazole was 0·2 ml. of a 0·3% ethanolic solution. *A*, 2:5-Diformylfuran (100 μg.); *B*, 5-formylfuroic acid (100 μg.); *C*, D-galacturonic acid (100 μg.); *D*, heparin (20 i.u.). Curve *A*, with carbazole, had $E_{max.}$ 3·2.

Galacturonic acid (5 mg.), after reaction at 60° and extraction with diethyl ether as described above for glucuronolactone at 100°, showed an absorption spectrum almost identical with that of an ethereal solution of 5-formylfuroic acid. The recovery was similar to that obtained with glucuronolactone at 100°.

DISCUSSION

That 5-formylfuroic acid is the principal light-absorbing product formed from uronic acids in the reactions in sulphuric acid at both 60° and 100° is indicated by (*a*) the almost identical forms of the absorption spectra of the compounds heated in sulphuric acid in the presence and absence of carbazole, (*b*) the higher molar absorption intensities shown by formylfuroic acid and (*c*) the absorption spectrum of a solution of 5-formylfuroic acid in diethyl ether having a maximum at 275 mμ identical in position with those shown by ether extracts of glucuronolactone in the 100° reaction and galacturonic acid in the 60° reaction. The

Table 2. *Intensities of absorption of light, and wavelength maxima, of substances in sulphuric acid in the reaction at* 60°, *with and without added carbazole*

Procedural details are described under Methods. Unless otherwise stated, 0·715 μmole of each substance was tested. Figures in parentheses refer to secondary absorption maxima. Figures in bold type refer to very low absorption intensities and are approximate.

Substance	Without carbazole, in the range 220–350 mμ		With carbazole, in the range 360–650 mμ	
	$\lambda_{max.}$	$E_{max.}$	$\lambda_{max.}$	$E_{max.}$
5-Formylfuroic acid	295	2·124	535 (405)	1·985 (0·773)
2:5-Diformylfuran	307 (222)	2·062 (1·120)	550 (440)	2·785 (1·250)
Furfuraldehyde	317 (256)	2·391 (0·763)	415 (525)	0·072 (**0·011**)
5-Hydroxymethylfurfuraldehyde	321 (258)	2·481 (0·555)	425 (537)	0·212 (0·203)
Galacturonic acid	296	0·548	532 (405)	0·568 (0·243)
Glucuronolactone	**298**	**0·026**	**530**	**0·009**
Glucuronic acid	**298**	**0·029**	**530**	**0·011**
Menthol glucuronoside	—	—	**530**	**0·008**
Methyl β-D-glucofuranuronolactone	296	0·048	530 (**405**)	0·050 (**0·021**)
1:2-*O*-*iso*Propylidene-α-D-glucofuranuronolactone	**298**	**0·024**	**530**	**0·011**
Xylose	317 (256)	2·330 (0·720)	425 (525)	0·058 (**0·010**)
Glucose	324 (257)	0·290 (0·068)	430 (550)	**0·012** (**0·009**)
Oxidized cellulose (100 μg.)	296	0·155	530 (405)	0·104 (0·070)
Chondroitin sulphate (310 μg.)	296	0·058	**540**	**0·039**
Heparin (20 i.u.)	297	0·141	530 (405)	0·142 (0·067)

formation of formylfuroic acid from uronic acids is analogous, on structural grounds, to the formation of furfuraldehyde from pentoses, of 5-hydroxymethylfurfuraldehyde from hexoses, and of furan-2:5-dicarboxylic acid from the saccharic acids during treatment of the appropriate compound with a strong acid (for a review see Newth, 1951). Decarboxylation, which occurs when uronic acids are boiled with hydrochloric acid (Lefevre & Tollens, 1907) and with 25–27% sulphuric acid (Meller, 1954), may be responsible, in part, for the difference between the absorption curves for formylfuroic acid and the uronic acids in the ultraviolet region in the reaction at 100° without added carbazole. The principal absorption maximum for glucuronolactone and for galacturonic acid is between those for formylfuroic acid and furfuraldehyde, which is one of the products of the decarboxylation reaction. Under the milder conditions of the reaction at 60° it appears probable that formylfuroic acid is the only product of the reaction of uronic acids with sulphuric acid which absorbs light in the ultraviolet region. It is therefore probably the only furan derivative formed in any appreciable quantity. The E values at $\lambda_{max.}$ suggest that conversion of galacturonic acid into formylfuroic acid in the reaction conducted at 60° is 25·8%. If it is assumed that formylfuroic acid is the only material (among the products of the reaction at 100°) that absorbs light in the ultraviolet region, conversion of glucuronolactone into formylfuroic acid is 59%, and of galacturonic acid 46%. It is probable, however, that at 100° some furfuraldehyde is also formed.

The amount of glucuronic acid in 100 μg. of oxidized cellulose or heparin is certainly considerably less than 0·715 μmole (for estimates of the actual content, by using the carbazole reaction at 100°, see Bowness, 1957), yet both of these compounds gave a much stronger reaction at 60° than does the equivalent amount of glucuronic acid or of the derivatives listed in Table 2. The absorption curves obtained with these two polysaccharides differed little from those of formylfuroic acid and galacturonic acid. It appears that some types of linkage (e.g. β-1:4 as in oxidized cellulose and to a lesser extent in the relatively unstable methyl β-glucofuranolactone) but not others (e.g. α-1:4 as in glucuronosides and 1:2-monoacetone glucofuranolactone) can increase the reactivity of glucuronic acid in the reaction at 60°.

SUMMARY

1. The absorption spectra in sulphuric acid, with and without carbazole, of 5-formylfuroic acid, 2:5-diformylfuran, furfuraldehyde, 5-hydroxymethylfurfuraldehyde, furan-2:5-dicarboxylic acid and the uronic acids indicate that 5-formylfuroic acid is responsible for colour formation in the carbazole procedures for estimating uronic acids.

2. 5-Formylfuroic acid appears to be the principal material absorbing ultraviolet light formed under the conditions examined.

3. 2:5-Diformylfuran also gives a colour, with a different absorption spectrum, under the same conditions.

4. Glucuronic acid in oxidized cellulose and heparin gives 5-formylfuroic acid more readily than the uncombined material.

I am grateful to Mr Lim Yen Heng for assistance with the preparation of 5-formylfuroic acid and 2:5-diformylfuran.

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Biochemical Studies of Toxic Agents

11. THE OCCURRENCE OF PREMERCAPTURIC ACIDS*

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(*Received* 28 *February* 1958)

Baumann & Preusse (1879) and Jaffé (1879) showed that the mercapturic acids which they isolated from the urine of dogs dosed with bromobenzene or chlorobenzene were formed by the decomposition of acid-labile compounds in the urine, and they believed that these precursors were derivatives of glucuronic acid. This work has recently been revised and extended as a result of the findings of Boyland, Sims & Solomon (1957) and Knight & Young (1957). From the urine of rabbits dosed with naphthalene, Boyland *et al.* (1957) and Boyland & Sims (1958) isolated a compound which on acidification yielded 1- and 2-naphthol as well as 1-naphthylmercapturic acid, and they suggested that the compound is *N*-acetyl-*S*-(2-hydroxy-1:2-dihydro-1-naphthyl)-L-cysteine. They also stated that anthracene and bromobenzene appear to give similar metabolites.

The present paper contains an account of chromatographic studies which have shown that the administration of benzene, naphthalene, anthracene, fluorobenzene, chlorobenzene, bromobenzene and iodobenzene to rabbits or rats is followed by the excretion of acid-labile precursors of the corresponding mercapturic acids. In a preliminary account of this work (Knight & Young, 1957) the name 'premercapturic acid' was proposed for such precursors. The radiochromatographic examination of urine containing ^{35}S-labelled 1-naphthylpremercapturic acid or *p*-bromophenylpremercapturic acid has indicated that acid decomposition of a premercapturic acid can lead to the formation of another sulphur-containing compound in addition to the mercapturic acid. Furthermore, by means of isotope-dilution techniques it has been possible to show that the amount of 1-naphthylmercapturic acid which can be liberated by mineral acid in the urine of rats dosed with naphthalene depends on the pH at which the 1-naphthylpremercapturic acid breaks down. Not all the mercapturic acids isolated from urine are derived from the decomposition of premercapturic acids, for chromatographic studies and tracer-isotope experiments have led to the conclusion that the urine of rats dosed with benzyl chloride does not contain an acid-labile precursor of benzylmercapturic acid.

* Part 10: Marsden & Young (1958).

CHROMATOGRAPHIC STUDIES

Reference compounds

Phenylmercapturic acid and 1-naphthylmercapturic acid. These non-radioactive compounds were obtained by the methods described by Marsden & Young (1958) for the ^{35}S-labelled compounds.

1-*Anthrylmercapturic acid.* The method of isolation of this compound was based on that used by Boyland & Levi (1936).

p-*Fluoro-*, p-*chloro-*, p-*bromo- and* p-*iodo-phenylmercapturic acid.* Methods similar to those described by Zbarsky & Young (1943) and Young & Zbarsky (1944) were used to isolate these compounds. In one experiment *p*-bromophenylmercapturic acid was extracted from acidified urine with 1:2-dichloroethane instead of with chloroform and this procedure had certain advantages. *p*-Fluorophenylmercapturic acid was isolated from the acidified urine of rats and rabbits dosed with fluorobenzene. The isolation of this compound from rabbit urine has not been reported hitherto.

Benzylmercapturic acid. S-Benzyl-L-cysteine was prepared by the reaction between benzyl chloride and L-cysteine in alkaline solution under conditions based on those used by Armstrong & Lewis (1951). The compound was acetylated with acetic anhydride in NaOH as described by Zbarsky & Young (1944) for the acetylation of *S*-(*p*-fluorophenyl)-L-cysteine.

Detection of premercapturic acids and mercapturic acids

Various methods have been used to detect mercapturic acids on paper chromatograms. Azouz, Parke & Williams (1955) employed a buffered acid–base indicator system for detecting certain of the mercapturic acids, a procedure described by Fewster & Hall (1951) for the detection of carboxylic acids. Boyland & Solomon (1956) detected 1-naphthylmercapturic acid on paper chromatograms by virtue of the fact that its fluorescence in ultraviolet light changed from pink to orange when the paper was sprayed with ammonia solution and heated. Bray, James & Thorpe (1956), in their studies of the mercapturic acids derived from the chloromononitrobenzenes, found that the ammonia in the solvent used for developing chromatograms of some of these mercapturic acids caused the acids to appear yellow on the paper.

A search for a general method of detecting premercapturic acids and mercapturic acids led us to the finding that quantities of the order of 5μg. of these compounds can be located by spraying the dried chromatograms with 0·1M-$K_2Cr_2O_7$–acetic acid (1:1) and then with 0·1M-$AgNO_3$.

After this treatment all the premercapturic acids and mercapturic acids tested appear as orange spots against a red–brown background. Chloride, which gives rise to a colourless area, is also detected by this reagent. Once the chromatograms have been sprayed it is important to protect them from the action of light, otherwise metallic silver is formed and obscures the spots.

Thirty-two compounds containing bivalent sulphur were tested with the reagent and gave positive reactions. (We are grateful to Dr G. A. Maw and Mr A. E. R. Thomson for samples of certain of these compounds.) In addition to the premercapturic acids and the mercapturic acids used in the present investigation, compounds tested included L-cystine, L-cysteine, a number of *S*-alkyl- and *S*-aryl-L-cysteines and several *S*-substituted thioglycollates. Several compounds containing sulphur in a higher oxidation state, including methionine sulphoxide, taurine and L-cysteic acid, gave negative reactions, as did a number of compounds which contained no sulphur. It is known, however, that purines will react with this reagent, although in a different way (Reguera & Asimov, 1950).

Animal experiments

Male rabbits and rats were used. The rabbits received a diet of rat cakes [J. Murray and Sons (London) Ltd.] and cabbage; the rats were given rat cakes only. The animals had access to water at all times and were housed in metabolism cages which permitted the collection of urine separate from faeces. Urine was collected daily and stored in the refrigerator.

Chromatograms of the urine of animals which had received benzene, naphthalene, anthracene, fluorobenzene, bromobenzene and benzyl chloride were developed by the descending method at 5°. Chromatograms of the urine of animals which had been dosed with chlorobenzene and iodobenzene were developed by the ascending method at room temperature. Whatman no. 1 paper was used throughout and chromatograms were developed in the upper phase of the system butan-1-ol–3N-$(NH_4)_2CO_3$–aq. 3N-NH_3 soln. (4:1:1). To see whether acid-labile premercapturic acids could be detected in the urine of the dosed animals a series of eight spots was applied to each 5 in. wide strip. These were: (i) and (ii), urine from dosed animals; (iii) and (iv), reference mercapturic acid; (v) and (vi), normal urine; (vii) and (viii), normal urine plus reference mercapturic acid. The second spot of each pair was treated with about 1·5 μl. of conc. HCl, the excess of which was removed in a current of warm air.

Results

Typical R_F values for the materials detected in the urines before and after acidification, together with the values for the mercapturic acids, are shown in Table 1. It will be seen that after the administration of every compound except benzyl chloride, the urine contained no compound with an R_F value equal to that of the corresponding mercapturic acid. A compound with a lower R_F value was present, however, and this was replaced on acidification by a compound with an R_F value equal to that of the corresponding mercapturic acid. The R_F value of the compound present in the urine of rabbits and rats dosed with benzyl chloride was similar to that of benzylmercapturic acid and was unchanged after acidification. Insofar as a urinary precursor of benzylmercapturic acid might have had the same R_F value as the mercapturic acid itself, these experiments were equivocal. Tracer-isotope experiments reported below, however, support the conclusion that no precursor of benzylmercapturic acid is present in the urine of rats which have been dosed with benzyl chloride.

Table 1. *R_F values of premercapturic acids and mercapturic acids*

Chromatograms of the unacidified urine and the acidified urine of the dosed animals were developed in the upper phase of the system butan-1-ol–3N-$(NH_4)_2CO_3$–aq. 3N-NH_3 soln. (4:1:1) by the descending method, except for those marked with an asterisk, for which the ascending method was used. Chromatograms were sprayed with 0·1M-$K_2Cr_2O_7$–acetic acid (1:1) followed by 0·1M-$AgNO_3$. Anthracene was administered by feeding the rats on a diet containing 4% (w/w) of the hydrocarbon (D). All other compounds were administered by stomach tube (ST) or by subcutaneous injection (SC).

Compound administered	Animal	Route	Dose (g./kg. body wt.)	Reference mercapturic acid	R_F values: Reference mercapturic acid	R_F values: Unacidified urine	R_F values: Acidified urine
Benzene	Rat	ST	1·0	Phenyl-	0·33	0·09	0·31
Naphthalene	Rat	SC	1·0	1-Naphthyl-	0·51	0·25	0·47
	Rabbit	ST	1·0		0·51	0·25	0·51
Anthracene	Rat	D	—	1-Anthryl-	0·54	0·39	0·55
Fluorobenzene	Rat	SC	1·0	*p*-Fluorophenyl-	0·41	0·19	0·38
	Rabbit	ST	1·0		0·41	0·20	0·42
Chlorobenzene	Rat	SC	1·0	*p*-Chlorophenyl-	0·51*	0·33*	0·50*
Bromobenzene	Rat	SC	1·0	*p*-Bromophenyl-	0·51	0·32	0·53
	Rabbit	ST	0·5		0·51	0·28	0·50
Iodobenzene	Rat	SC	1·0	*p*-Iodophenyl-	0·50*	0·29*	0·47*
Benzyl chloride	Rat	SC	1·0	Benzyl-	0·36	0·35	0·35
	Rabbit	SC	0·2		0·34	0·31	0·32

Evidence that 1-naphthylmercapturic acid was formed from the compound in the unacidified urine which gave an orange-coloured spot with the $K_2Cr_2O_7$–$AgNO_3$ reagent was obtained from the following experiment. Three spots, each containing 20 μl. of the urine of rats dosed with naphthalene, were applied to a length of Whatman no. 1 paper and development was carried out in butan-1-ol saturated with aq. 3N-NH_3 soln. Ammonium carbonate was not included in the solvent system in this part of the experiment, in order to exclude this salt from the material which was to be eluted subsequently. Its absence had only a slight effect on the R_F values of the compounds detected. The developed chromatogram was cut into three 1 in. wide strips, one of which was sprayed with the $K_2Cr_2O_7$–$AgNO_3$ reagent and the compound giving a positive reaction was thereby located. The corresponding areas on the other two strips were cut out and together were macerated with 1 ml. of water. The suspension of cellulose fibres was filtered on a sintered funnel and the cellulose was resuspended in 0·5 ml. of ethanol. The ethanol and aqueous extracts were dried together in a current of warm air and the residue was dissolved in a mixture of 0·1 ml. of water and 0·1 ml. of ethanol. The resulting solution was applied to a 3 in. wide strip of Whatman no. 1 chromatographic paper so as to form two spots, and to one of these was added about 1·5 μl. of conc. HCl. A third spot containing 15 μg. of 1-naphthylmercapturic acid was formed on the paper. The chromatogram was developed in the upper phase of the system butan-1-ol–3N-$(NH_4)_2CO_3$–aq. 3N-NH_3 soln. (4:1:1) and was sprayed with the $K_2Cr_2O_7$–$AgNO_3$ reagent. The spot which had been treated with HCl yielded a compound with the same R_F value as 1-naphthylmercapturic acid, whereas the untreated spot gave rise to material which corresponded to that eluted from the first chromatogram.

TRACER–ISOTOPE STUDIES

Measurement of ^{35}S. Compounds containing radioactive sulphur were oxidized by the Carius procedure and ^{35}S was counted as benzidine sulphate as described previously (Hawkins & Young, 1954; Marsden & Young, 1958).

[^{35}S]*Yeast.* When yeast is grown in a medium containing inorganic [^{35}S]sulphate to incorporate labelled sulphur into sulphur-containing amino acids of the yeast (Williams & Dawson, 1952), a considerable proportion of the [^{35}S]-sulphate remains in the medium after the yeast has been harvested. It was found that by adding further quantities of the original constituents to the culture medium several crops of yeast could be grown and by this means the [^{35}S]-sulphate was more economically used.

The yeast was separated from the medium by centrifuging and was washed three times in the centrifuge bottles, twice with 95% ethanol and once with absolute ethanol. It was finally dispersed in ether and filtered on a Büchner funnel. After this treatment it was very powdery and was handled as far as possible in a glove box. The yeast preparation obtained in this way is described in the present paper as [^{35}S]yeast.

L-[^{35}S]*Cystine.* This was isolated from [^{35}S]yeast by the method of Williams & Dawson (1952) with the minor modifications introduced by Marsden & Young (1958).

^{35}S-*Labelled mercapturic acids*

Three methods were used to prepare mercapturic acids labelled with ^{35}S. Those required as reference compounds for radiochromatography were prepared from the urine of rats which had received the appropriate parent compound and [^{35}S]yeast. The 1-naphthylmercapturic acid which was used in the isotope-dilution experiments was prepared from the urine of rats which had received naphthalene and L-[^{35}S]cystine. The method used to prepare the [^{35}S]benzylmercapturic acid employed in the isotope-dilution experiments was based on the reaction between benzyl chloride and L-[^{35}S]cysteine in anhydrous NH_3.

[^{35}S]1-*Naphthylmercapturic acid.* This compound was obtained from the urine of rats dosed with naphthalene and L-[^{35}S]cystine by the method described by Marsden & Young (1958). It had a specific activity of 2·86 μc/m-mole.

[^{35}S]1-Naphthylmercapturic acid of much higher specific activity was obtained by giving the rats [^{35}S]yeast instead of L-[^{35}S]cystine. Two rats were fasted overnight and then each was given by stomach tube 2 g. of [^{35}S]yeast suspended in water. On the next day the rats were each given 1·5 g. of the [^{35}S]yeast and at the same time each was injected subcutaneously with 0·2 g. of naphthalene as a 20% (w/v) solution in arachis oil. In order to obtain the mercapturic acid and also a urine fraction containing the premercapturic acid, the following procedure was used. A volume (10 ml.) of the urine voided during the 24 hr. after the second dose of [^{35}S]yeast was treated with 10 ml. of ethanol. The mixture was centrifuged and the supernatant liquid, after evaporation under reduced pressure, yielded a gum. This was stirred with 10 ml. of ethanol to which was added the minimal amount of water to make solution of the gum almost complete. A large excess of ethanol was then added with stirring and, after the mixture had been chilled, the precipitate which had formed was separated by centrifuging in the refrigerator. The supernatant liquid was evaporated to a gum which was treated with butan-1-ol saturated with aq. 0·3N-NH_3 soln. and then with just sufficient ethanol and water to bring it into solution. This solution was chromatographed on a column (45 cm. × 3 cm.) containing cellulose powder. The column had been prepared from a slurry of cellulose in butan-1-ol saturated with aq. 0·3N-NH_3 soln. This solvent was also used for development and elution. The appearance of ^{35}S in the eluate was detected by allowing a drop of each fraction to dry on a filter paper and then examining the paper for the presence of radioactivity. Spots which contained ^{35}S also gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent. The fractions containing ^{35}S were pooled and evaporated under reduced pressure to a gum which was dissolved in 5 ml. of ethanol. A volume (1 ml.) of this solution was reserved for radiochromatographic examination. To the other 4 ml. was added 12 ml. of water and 1·5 ml. of conc. HCl; the resulting suspension was cooled for 2 days and yielded 0·016 g. of grey crystals. These were dissolved in ethanol and the solution was treated with activated charcoal and filtered. The addition of hot water to the filtrate caused the precipitation of a white crystalline material with m.p. 170° (m.p. values reported in this paper are uncorrected) unchanged by admixture with a sample of non-radioactive 1-naphthylmercapturic acid. The material had a specific activity of 254 μc/m-mole.

[^{35}S]p-*Bromophenylmercapturic acid*. Two rats were each given by stomach tube 1·4 g. of [^{35}S]yeast and at the same time each was injected subcutaneously with bromobenzene (1 g./kg. body wt.). The urine was collected for the next 24 hr. and was treated in a manner similar to that described for the urine of rats dosed with naphthalene, in order to obtain the mercapturic acid and also a fraction containing the premercapturic acid. After purification of the precipitate formed by acidification of part of the urine fraction containing the premercapturic acid, the product obtained had m.p. 154·5–155° and mixed m.p. 154·5–155·5° with a sample of non-radioactive *p*-bromophenylmercapturic acid.

[^{35}S]*Benzylmercapturic acid*. Two rats were dosed by stomach tube with an aqueous suspension of [^{35}S]yeast on 2 consecutive days, and on the second day each rat was injected subcutaneously with benzyl chloride (1 g./kg. body wt.). During the next 24 hr., 16 ml. of urine was collected. Some of this urine was used for radiochromatography and the rest (14 ml.) was treated with 1·4 ml. of conc. HCl and extracted by shaking three times with 15 ml. portions of ethyl acetate. The combined extracts were evaporated under reduced pressure and yielded a gum. When this was warmed with 2 ml. of water and stirred vigorously, most of it dissolved. The supernatant liquid was decanted from a small amount of undissolved material and was then cooled. The crystalline precipitate which formed was filtered off and treated in aqueous solution with charcoal. The suspension was filtered and the filtrate deposited white crystals (0·032 g.), m.p. 146–147°.

[^{35}S]Benzylmercapturic acid was synthesized by the following method. L-[^{35}S]Cystine (0·962 g.) was dissolved in about 100 ml. of anhydrous NH_3 contained in a flask surrounded by a mixture of solid carbon dioxide and ethanol. Small, freshly cut portions of sodium metal were added while the reaction mixture was vigorously stirred, until a total of 0·4 g. had been introduced. The reduction was taken as complete when a blue colour persisted in the reaction mixture for a short time. Benzyl chloride (1·1 ml.) was then added drop by drop until a nitroprusside test showed the absence of –SH groups. The mixture was stirred for 10 min. and the NH_3 was then allowed to evaporate. The ^{35}S-labelled *S*-benzyl-L-cysteine was acetylated with acetic anhydride in alkaline solution by the method used for the non-radioactive compound. After crystallization from water and treatment with charcoal the product had a specific activity of 11·9 μc/m-mole.

Radiochromatographic experiments

The observations made from paper-chromatographic studies were confirmed and extended by radiochromatographic experiments. This work included the examination not only of urine, but also of urine fractions prepared as described earlier in the paper. The object of the fractionation was to

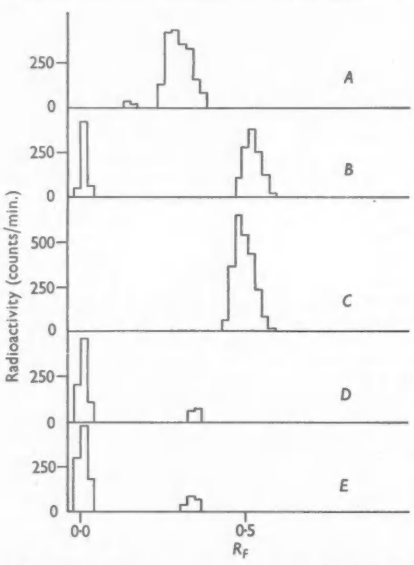


Fig. 1. Naphthalene metabolism. Radiochromatograms developed in the upper phase of the system butan-1-ol–3N-$(NH_4)_2CO_3$–aq. 3N-NH_3 soln. (4:1:1). *A*, Unacidified and *B*, acidified fraction from urine of rats dosed with naphthalene and [^{35}S]yeast; *C*, [^{35}S]1-naphthylmercapturic acid; *D*, unacidified and *E*, acidified urine of rats dosed with [^{35}S]yeast.

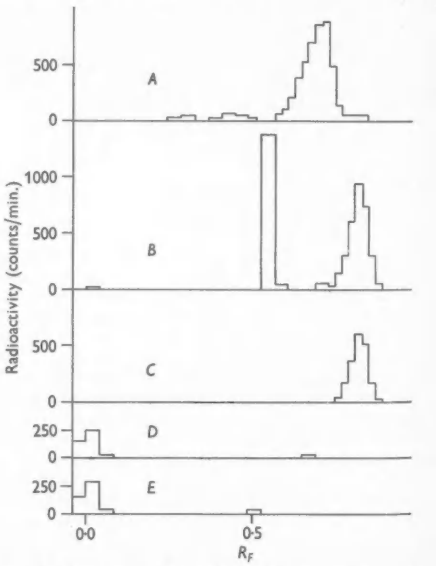


Fig. 2. Naphthalene metabolism. Radiochromatograms developed in the upper phase of the system butan-1-ol–saturated aq. sodium acetate–acetic acid–water (80:3:1:36). *A*, *B*, *C*, *D* and *E* as in Fig. 1. The sharpness of the peak with the lower R_F value in *B* arises from the fact that this material travels at the front of the area containing chloride.

free the premercapturic acids from other radioactive constituents, the presence of which complicated the interpretation of the radiochromatograms.

Methods. Radiochromatograms were developed at room temperature by the ascending method on 3 in. wide Whatman no. 1 paper. The two solvent systems used were the upper phases of the systems butan-1-ol–3 N-$(NH_4)_2CO_3$–aq. 3 N-NH_3 soln. (4:1:1) and butan-1-ol–saturated aq. sodium acetate soln.–acetic acid–water (80:3:1:36). Two spots of material to be chromatographed were applied to each paper, each spot being 1 in. from the edge. After development the papers were dried in the air and sprayed with the $K_2Cr_2O_7$–$AgNO_3$ reagent. Strips 1 in. wide were then cut from the chromatograms so that each included all the material derived from a single spot. Each strip was then marked in intervals of 0·5 cm. along its length and scanned for the presence of radioactivity with an apparatus described by Lowenstein (1953; see also Corner & Young, 1955). In the present work the radioactivity of each 0·5 cm. section was counted for at least 1 min. Although the background count varied somewhat when measured over 1 min. only, it was generally about 25 counts/min. It did not exceed 40 counts/min. and the graphical representation of the data has been simplified by excluding activities below this figure.

Experiments with naphthalene. Because of the higher activity of the sulphur-containing compounds present, it was found that the urine excreted after giving [^{35}S]yeast and naphthalene to rats was more suitable for radiochromatographic examination than that obtained after giving L-[^{35}S]cystine with the naphthalene. The urine of rats which had received [^{35}S]yeast alone gave rise to no areas of radioactivity on the chromatograms which might have been confused with the mercapturic acid or its precursor. Diagrammatic representations of the radiochromatograms developed in two solvent systems from the urine fraction from rats dosed with naphthalene, before and after acidification, are shown in Figs. 1 and 2. These diagrams show that a radioactive component which is present in the urine is replaced on acidification by two components, one with a higher R_F value and the other with a lower R_F value than the original component. There seems little doubt that the faster-moving component is 1-naphthylmercapturic acid, not only on account of its R_F value, but also because this compound was isolated from the urine fraction used in the radiochromatographic experiments.

Experiments with bromobenzene. The diagrams in Fig. 3 are of the radiochromatograms developed from the urine fraction before and after acidification. The results obtained are similar to those just described for experiments with naphthalene in that a radioactive compound gives rise to two radioactive components on acidification, one of which is the mercapturic acid.

Experiments with benzyl chloride. Fig. 4 shows the radiochromatograms obtained from the urine

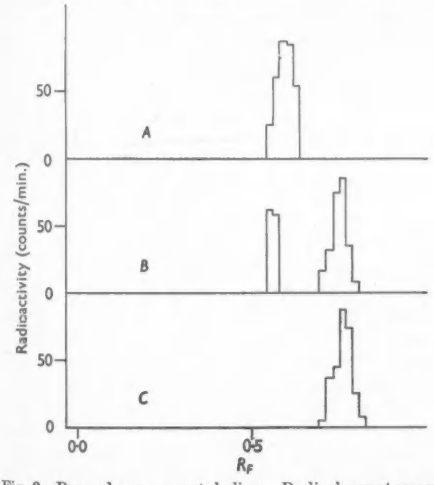


Fig. 3. Bromobenzene metabolism. Radiochromatograms developed in the upper phase of the system butan-1-ol–saturated aq. sodium acetate–acetic acid–water (80:3:1:36). *A*, Unacidified, and *B*, acidified fraction from urine of rats dosed with bromobenzene and [^{35}S]-yeast; *C*, [^{35}S]*p*-bromophenylmercapturic acid. The sharpness of the peak with the lower R_F value in *B* arises from the fact that this material travels at the front of the area containing chloride.

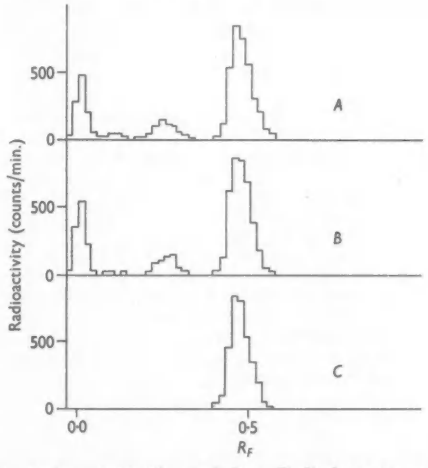


Fig. 4. Benzyl chloride metabolism. Radiochromatograms developed in the upper phase of the system butan-1-ol–3 N-$(NH_4)_2CO_3$–aq. 3 N-NH_3 soln. (4:1:1). *A*, Unacidified, and *B*, acidified urine of rats dosed with benzyl chloride and [^{35}S]yeast; *C*, [^{35}S]benzylmercapturic acid.

before and after acidification. These gave no evidence for the presence of a precursor of benzylmercapturic acid in the urine.

Isotope-dilution experiments

The availability of ^{35}S-labelled mercapturic acids made possible the determination of the unlabelled compounds by an isotope-dilution technique. This was used in experiments in which portions of the urine of rats which had been dosed with naphthalene or benzyl chloride were kept under different conditions of acidity for known lengths of time. A known amount of the ^{35}S-labelled mercapturic acid in the form of its ammonium salt was added to the portion of urine after adjustment to pH 8·0, and the radioactive mercapturic acid together with any non-radioactive mercapturic acid which was present in the urine was then extracted at pH 5·0. From a comparison of the specific activity of the added mercapturic acid and that isolated it was possible to calculate the amount of mercapturic acid present in the treated urine.

During the process of acidification and also when the urine was subsequently being made alkaline, great care was taken to reduce to a minimum the occurrence of local concentrations of acid or alkali. The urine was adjusted to pH 8·0 for the addition of the ^{35}S-labelled mercapturic acid in order to ensure the complete mixing of this with the non-radioactive mercapturic acid. All the extractions of the mercapturic acids were carried out under the same conditions of acidity and each portion was adjusted to pH 5·0 and buffered at this pH immediately before extraction. The samples of mercapturic acid isolated from the urine were purified to constant specific activity.

Occurrence of 1-naphthylpremercapturic acid in the urine of rats dosed with naphthalene. While lightly anaesthetized with ether, 12 rats were each dosed by subcutaneous injection with 0·5 ml. of 20% (w/v) naphthalene in arachis oil at each of two dorsal sites. The 24 hr. urine (110 ml., pH 6·4) was diluted with water to 250 ml. and four 50 ml. portions were treated as follows.

Portion A. This was further diluted with 20 ml. of water and stirred vigorously while it was adjusted to pH 5·0 by the slow addition of 0·25 ml. of 2N-HCl. Water (4·75 ml.) was then added to bring the total volume to 75 ml. The solution was adjusted to pH 8·0 by the addition of 0·8 ml. of 2N-NaOH, and 0·0925 g. of [^{35}S]1-naphthylmercapturic acid which had been dissolved in a small amount of aq. 2N-NH_3 soln. was added. The solution was again stirred vigorously while it was adjusted to pH 5·0 by the slow addition of 1·2 ml. of 2N-HCl. After the addition of 20 ml. of phosphate–citrate buffer at pH 5·0 (McIlvaine series; Vogel, 1951) the volume of the solution was adjusted to 110 ml. with water. The solution was next extracted by shaking for 15 min. with each of four 110 ml. portions of chloroform. The combined chloroform extracts were evaporated to a small volume, which was transferred to a 30 ml. beaker in which evaporation was completed in a current of air. The residue (0·062 g.) was stirred with 2 ml. of chloroform, cooled in ice and filtered off, by which process most of the colouring material was removed. The residue was dissolved in 6 ml. of ethanol and warmed with charcoal. The suspension was filtered and the filter paper was washed with 2 ml. of ethanol. The filtrate and washings were combined and evaporated in a current of air to 4 ml. The solution was warmed, 16 ml. of hot water was added, and a crystalline precipitate separated. After the mixture had been cooled overnight in the refrigerator it was filtered on a sintered funnel and the residue was suspended in 2 ml. of ether and then sucked dry. This process yielded 0·044 g. of material, which was purified to constant specific activity by three crystallizations from ethanol–water (20:80, v/v). The weight of non-radioactive 1-naphthylmercapturic acid present in the portion of urine was calculated from the difference in specific activity of the [^{35}S]1-naphthylmercapturic acid added to the urine and that of the mercapturic acid isolated from the urine.

Portion B. To this was added 5 ml. of 2N-HCl in order to bring it to pH 1·0, after which its volume was adjusted to 75 ml. by the addition of water. After 5 min. the solution was adjusted to pH 8·0 by the addition of 6·7 ml. of 2N-NaOH. [^{35}S]1-Naphthylmercapturic acid (0·1088 g.) was dissolved in a small volume of aq. 2N-NH_3 soln. and added to the urine, followed by 1·4 ml. of 2N-HCl which brought the solution to pH 5·0. Phosphate–citrate buffer, pH 5·0 (20 ml.), was next added and the volume of the solution was adjusted to 110 ml. with water. The resulting solution was extracted with chloroform and the extract was treated in a similar manner to the extract of portion A.

Portion C. This was treated in the same way as portion B except that the solution was kept at room temperature at pH 1·0 for 24 hr. instead of for 5 min.

Portion D. This was treated with 7·5 ml. of conc. HCl followed by 17·5 ml. of water and left at room temperature for 24 hr. It was then adjusted to pH 8·1 by the addition of 9 ml. of 40% (w/v) NaOH solution, and 0·1075 g. of [^{35}S]1-naphthylmercapturic acid which had been dissolved in a small volume of aq. 2N-NH_3 soln. was added. The addition of 1·5 ml. of 2N-HCl brought the solution to pH 5·0 and 20 ml. of phosphate–citrate buffer, pH 5·0, was then added. The extraction with chloroform and the purification of the extract were carried out as described for portion A.

The results of this experiment (Expt. 1) and of another (Expt. 2) carried out in the same way are shown in Table 2. It will be seen that when the acidity of a portion of the urine of rats dosed with naphthalene was not greater than pH 5·0, the amount of 1-naphthylmercapturic acid detected was less than 2% of that found in another portion which had been treated with one-tenth of its volume of conc. HCl. While a small amount of the mercapturic acid may have been formed after the urine had been excreted, the urine kept at pH 5·0 may, nevertheless, have contained no free mercapturic acid, for the small quantity found lies within the limits of experimental error.

The data in Table 2 suggested that the method of breakdown of 1-naphthylpremercapturic acid might

Table 2. *Occurrence of* 1-*naphthylpremercapturic acid in the urine of rats dosed with naphthalene*

Twelve rats each received 0·2 g. of naphthalene and the urine excreted in the following 24 hr. was used. Results are shown for two experiments in which an isotope-dilution technique was used to determine the amounts of 1-naphthylmercapturic acid present in portions of the urine which had been kept at room temperature under different conditions of acidity for known lengths of time. Each portion represented one-fifth of the 24 hr. urine. For further experimental details see text.

Portion	Acidity	Duration	1-Naphthylmercapturic acid found (mg.) Expt. 1	Expt. 2
A	pH 5·0	0	1	1
B	pH 1·0	5 min.	9	6
C	pH 1·0	24 hr.	20	38
D	Normal to HCl	24 hr.	59	78

Table 3. *Influence of* pH *on the breakdown of* 1-*naphthylpremercapturic acid*

Twenty-four rats each received 0·2 g. of naphthalene and the urine excreted in the following 24 hr. was used. Results are shown for experiments in which an isotope-dilution technique was used to determine the amounts of 1-naphthylmercapturic acid present in portions of the urine which had been kept at 30° for known lengths of time at pH 1·0 or normal to HCl. Each portion represented one-tenth of the 24 hr. urine. For further experimental details see text.

Duration (hr.)	1-Naphthylmercapturic acid found (mg.) pH 1·0	Normal to HCl
0	2	—
2	18	58
6	18	—
24	19	67
72	19	62

be dependent on the pH at which the decomposition occurs, and this possibility was investigated in the following experiment.

Influence of pH *on the breakdown of* 1-*naphthylpremercapturic acid.* This experiment was designed to enable an investigation to be made of the maximal amounts of 1-naphthylmercapturic acid which could be formed under two conditions of acidity in the urine of rats dosed with naphthalene. To this end twenty-four rats were injected with naphthalene as previously described. The 24 hr. urine was collected and its volume adjusted to 500 ml. by the addition of water. Eight 50 ml. portions of the diluted urine were treated as follows. Four were adjusted to pH 1·0 and kept at 30° for periods of 2, 6, 24 and 72 hr., and three other portions were made normal to HCl and kept at 30° for periods of 2, 24 and 72 hr. At the end of these periods they were brought to pH 5·0 and a solution of 0·1 g. of [^{35}S]1-naphthylmercapturic acid as its ammonium salt was added to each. Each solution was cooled in ice, adjusted to pH 1·0 and was shaken for 10 min. with twice its volume of chloroform. The 1-naphthylmercapturic acid was then isolated from the chloroform extract and its specific activity measured as already described. The eighth portion of the urine was brought to pH 5·0, 0·1 g. of [^{35}S]1-naphthylmercapturic acid as the ammonium salt was added and the solution was cooled in ice. It was then adjusted to pH 1·0 and immediately extracted with chloroform and the chloroform extract was treated as already described. The amounts of 1-naphthylmercapturic acid found in the various portions of urine are shown in Table 3.

As it seemed likely that some breakdown of the mercapturic acid itself occurred under the conditions of the experiments just described, further experiments were carried out to determine the extent to which this might have taken place. Urine from normal rats was collected, a solution of 1-naphthylmercapturic acid was added to it and four portions were taken. Two were made to pH 1·0 and the other two were made normal to HCl. All four were incubated at 30° and the isotope-dilution method already described was used to determine the amount of 1-naphthylmercapturic acid present in each after 24 and 72 hr. The results showed that even after 72 hr. in N-HCl less than 13 % of the mercapturic acid originally present had been broken down.

Absence of a urinary precursor of benzylmercapturic acid in the urine of rats dosed with benzyl chloride. An experiment similar to that carried out with the urine of rats dosed with naphthalene was undertaken with the urine of rats dosed with benzyl chloride to examine the possibility, suggested by the chromatographic and radiochromatographic studies, that benzylmercapturic acid is excreted as such and not as an acid-labile precursor.

Each of eighteen rats was lightly anaesthetized with ether and injected subcutaneously with 0·075 ml. of benzyl chloride in each of two dorsal sites. During the subsequent 24 hr. the animals were allowed water but not food. The 24 hr. urine (206 ml., pH 6·4) was collected and four 50 ml. portions were treated as follows.

Portion A. This was treated with 2N-HCl, which was added slowly and with continuous stirring until the solution was at pH 5·0. In a similar manner 1·3 ml. of 2N-NaOH was added to adjust the solution to pH 8·0, and then 0·1270 g. of [^{35}S]benzylmercapturic acid which had been dissolved in 1 ml. of aq. 2N-NH_3 soln. was added. The solution was brought back to pH 5·0 by the addition of 1·7 ml. of 2N-HCl and water was added to make a total volume of 100 ml. After the addition of 20 ml. of phosphate–citrate buffer, pH 5·0, the urine was extracted four times by shaking each time for 15 min. with 120 ml. of ethyl acetate. The combined extracts were evaporated to a gum which was dissolved in 5 ml. of warm water. The crystalline precipitate which formed on leaving this solution overnight in the refrigerator was filtered off, dissolved in warm water and treated with charcoal, after which the suspension was filtered and the filtrate was again cooled in the refrigerator. The cold filtrate was filtered on a sintered funnel, and the residue was suspended in ether on the funnel and sucked dry. The residue was purified to constant specific activity by three further crystallizations from water. The amount of benzylmercapturic acid present in the urine was calculated from the difference in specific activity of the [^{35}S]benzylmercapturic acid added to the urine and that isolated.

Portion B. This was adjusted to pH 1·0 by the addition of 20 ml. of 2N-HCl and retained at this pH for 5 min. The addition of 24·2 ml. of 2N-NaOH brought the solution back to pH 8·0 and 0·1284 g. of [^{35}S]benzylmercapturic acid, as a solution of its ammonium salt, was added. The solution was then adjusted to pH 5·0 and adjusted to 100 ml. with water. Phosphate–citrate buffer, pH 5·0 (20 ml.), was added and the solution was then extracted with ethyl acetate. The extract was then purified in the manner described for portion A.

Portion C. This was treated in the same way as portion B except that it was kept at pH 1·0 for 24 hr.

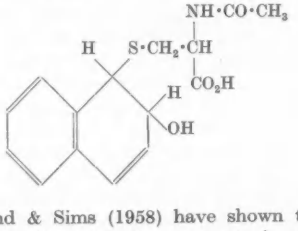
Portion D. This was treated with 7 ml. of conc. HCl and 13 ml. of water and kept at room temperature for 24 hr. It was then adjusted to pH 8·0 by the addition of 9·2 ml. of 40% (w/v) NaOH and this was followed by the addition of 0·1278 g. of [^{35}S]benzylmercapturic acid, as a solution of its ammonium salt. The solution was adjusted to pH 5·0 with 3 ml. of 2N-HCl and its volume was made to 100 ml. by the addition of water. After the addition of 20 ml. of phosphate–citrate buffer, pH 5·0, the solution was extracted and the extract was purified as described for portion A.

The amounts of benzylmercapturic acid found (expressed as percentages of benzyl chloride converted into the mercapturic acid) were as follows: A, 14; B, 14; C, 13; D, 14. These findings are quite different from those obtained in similar experiments with naphthalene (see Table 2) in that they indicate no significant increase in the amount of mercapturic acid in the urine after acidification.

DISCUSSION

The present work has provided evidence that the monohalogenobenzenes and certain cyclic hydrocarbons are converted in the animal body into pre-mercapturic acids, that is, into compounds which are broken down on acidification to yield mercapturic acids. The question arises of whether compounds which give rise to premercapturic acids are excreted in part as mercapturic acids. As chromatographic experiments did not indicate the presence of mercapturic acids together with premercapturic acids in unacidified urine, this matter was examined further by making use of an isotope-dilution technique. In these experiments the unacidified urine of rats dosed with naphthalene appeared to contain a small amount of 1-naphthylmercapturic acid, but this did not correspond to more than 0·1% of the naphthalene administered. While this matter requires further investigation, the lability of the premercapturic acids is such that this small amount of mercapturic acid may have been formed by breakdown of the precursor in the urine.

The labile nature of 1-naphthylpremercapturic acid immediately brings to mind similar characteristics shown by certain other metabolites of naphthalene. When Bourne & Young (1934) observed that the urine of rabbits which had received naphthalene yielded the hydrocarbon on acidification, they pointed out that this might be accounted for by the presence in the urine of a compound such as 1:2-dihydro-2-naphthol. The naphthalene precursor has since been shown to be 1:2-dihydro-1-naphthyl glucosiduronic acid (Boyland & Solomon, 1955). Two other derivatives of dihydronaphthalene have also been shown to be metabolites of naphthalene, namely 1:2-dihydronaphthalene-1:2-diol (Young, 1947; Booth & Boyland, 1949) and 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid (Corner, Billett & Young, 1954). From both of these compounds the elements of water are eliminated on acidification. Under these circumstances it seems reasonable to suppose that the premercapturic acid might also have a dihydronaphthalene structure with a hydroxy group on C-2, as shown below. This structure has been suggested by Boyland & Sims (1958) on the basis of their study of the isolated compound.



Boyland & Sims (1958) have shown that the breakdown of 1-naphthylpremercapturic acid under acid conditions gives rise to 1- and 2-naphthol as well as the mercapturic acid. In the present work

it has been shown that the amount of 1-naphthylmercapturic acid formed is dependent on the pH at which the breakdown occurs. This raises the interesting possibility that there may be some premercapturic acids which on acidification yield mainly hydroxy compounds and little or no mercapturic acid. For example, although studies of the influence of phenanthrene on sulphur metabolism (Elson, Goulden & Warren, 1945; Crabtree, 1945, 1946) have yielded results consistent with the formation of an acetylcysteine conjugate of this hydrocarbon, there have been no reports of the isolation of such a compound from the urine of the dosed animals. Although mercapturic acids have not been isolated from the urine of animals dosed with carcinogenic hydrocarbons, the isolation of hydroxy derivatives has been reported. Under these circumstances, the possibility that the hydroxy compounds are derived, in part at least, from the breakdown of premercapturic acids would seem to merit consideration.

An interesting aspect of the present work is that benzyl chloride appears to be converted directly *in vivo* into a mercapturic acid. This was first suggested by the results of chromatographic and radiochromatographic studies and was subsequently supported by evidence obtained from isotope-dilution experiments. It would seem therefore that there are some compounds which are metabolized to mercapturic acids and do not give rise to premercapturic acids. On the other hand, there are others which form premercapturic acids and in all probability are not metabolized to mercapturic acids.

SUMMARY

1. Paper-chromatographic methods and tracer-isotope techniques with ^{35}S-labelled compounds have been used in a study of the occurrence of premercapturic acids, i.e. acid-labile precursors of mercapturic acids.

2. It has been shown that the administration to animals of benzene, naphthalene, anthracene and the monohalogenobenzenes is followed by the excretion of the corresponding premercapturic acids.

3. The amount of 1-naphthylmercapturic acid which can be obtained by the breakdown of 1-naphthylpremercapturic acid under acid conditions has been shown to be dependent on the pH at which decomposition occurs.

4. Evidence has been obtained that in the animal body benzyl chloride is converted directly into a mercapturic acid and does not give rise to a premercapturic acid.

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Phenolic Acids and Flavonoids of *Theobroma cacao* L. Separation and Identification by Paper Chromatography

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Previous investigations on the phenolic constituents of *Theobroma cacao* L. have been largely limited to studies on the polyphenols of the ripe bean (Forsyth, 1952, 1955; Forsyth & Quesnel, 1957), which have been shown to be of major importance in determining the taste characteristics of processed cacao.

Studies are now being pursued in this Laboratory on the nature of the factors determining the chemical composition of the bean at maturity with particular reference to the phenolic constituents of the cotyledons. The physical and biochemical factors determining the level of polyphenols other than anthocyanins in vegetative and reproductive organs of the green plant are largely unknown, although it has recently been shown by Hillis & Swain (1957) that illumination is an important factor in the formation of the leaf leucocyanidins of *Prunus domestica* var. *victoria*. Other of our current investigations are concerned with the possible role of the phenols in the mechanism of disease resistance of *T. cacao*, evidence having been obtained of an intense metabolic activity involving the host polyphenols of *T. cacao* tissue after invasion by *Marasmius perniciosus* (L. A. Griffiths, unpublished work). For both these projects a systematic investigation of the phenolic constituents of both vegetative and reproductive tissues was a necessary preliminary and the results obtained are presented in this paper. Although there are many reports in the literature describing the sporadic occurrence of phenolic compounds in various plant materials, a study of the distribution and interrelations of this class of compound throughout the vegetative and reproductive structures of a single species of green plant does not appear to have been previously undertaken.

In this survey of the phenolic constituents of *T. cacao*, both methanolic and acid-hydrolysed extracts have been examined by paper chromatography.

MATERIALS AND METHODS

Source of plant material. Unless otherwise stated, all plant material was obtained from trees of *Theobroma cacao*, of the clone I.C.S. 1 (Imperial College Selection 1).

Extraction and separation

Phenolic acids of tissue hydrolysates. Finely chopped fresh tissue (5 g.) was heated with 20 ml. of aq. 2N-HCl for 20 min. on a boiling-water bath. The cooled filtrates were extracted with 5 ml. of diethyl ether and 0·1 ml. of the ethereal extract was applied to the chromatogram by repeated applications in a warm air-stream.

The chromatograms were developed by an ascending method employing benzene–acetic acid–water, 60:70:30 (solvent *A*) (Griffiths, 1957*a*). The original procedure was modified only in that the chromatograms were run at 20° instead of 15° as previously used. It was observed that the use of a higher temperature caused an appreciable rise in the R_F values recorded. Although this system was originally developed for the separation of the phenolic acids, it has also proved of utility in the preliminary identification of the flavonols found in these extracts. By extending the development time to 16 hr. and by the use of descending chromatography, flavonols of low R_F, such as quercetin, could be readily separated. Their presence was confirmed with the butanol–acetic acid–water solvent.

Free polyphenols. The polyphenols of *T. cacao* tissue were extracted by homogenizing 5 g. of fresh tissue in 25 ml. of cold absolute methanol in a Micro-Blender for 3 min. A volume (0·05 ml.) of the filtered extract was then applied to the chromatogram.

Both one- and two-dimensional chromatography were employed. With the former the solvent employed was butan-1-ol–acetic acid–water (40:10:50), and, with the latter, water was employed as the first solvent (solvent *C*), and the butanol–acetic acid–water system (solvent *D*) as the second. All two-dimensional work was carried out in a Shandon Multi-sheet Chromatank.

Anthocyanidins. A previously described extraction procedure and chromatographic method employing water–acetic acid–HCl, 10:30:3 (solvent *B*), (Bate-Smith, 1954), was employed. Identification of the anthocyanidin was in all cases confirmed by the use of markers of known identity.

Detection of phenolic compounds

Ultraviolet fluorescence. The hydroxycinnamic acids, their derivatives and the flavonols were detected by examination in ultraviolet light, when they fluoresced in a characteristic manner in the presence and absence of ammonia (Griffiths, 1957*a*).

Spray reagents. The *p*-nitroaniline-spray reagent previously employed for the detection of phenolic acids (Swain, 1953; Griffiths, 1957*a*) was found to be of value also in the detection of catechins and leucocyanidins, which gave rise

to yellow–brown coloration on spraying. The toluene-*p*-sulphonic acid reagent of Roux (1957) was employed for the detection of leucocyanidins.

R_F values. Since R_F values vary considerably with temperature, it was found essential under tropical conditions to effect direct comparison of the unknown compounds in each case with markers of known identity, although for the two-dimensional chromatograms the individual polyphenols are readily recognized by their position in the overall pattern of spots. The identity of the compounds marked with an asterisk in Table 2 was confirmed in each case by admixture of the extract with an authentic specimen of the compound, when intensification of the corresponding spot was observed.

RESULTS

Phenolic acids and flavonoids of tissue hydrolysates

The general distribution of the phenolic acids in the tissues of *T. cacao* is shown in Table 1. Caffeic acid and a compound of slightly higher R_F (0·28 at 20°), exhibiting a green–blue fluorescence in u.v. light, were found to be of widespread occurrence. Other fluorescent phenols detected appeared to be restricted to tissues of a specific morphological type.

Fruit. The tissues of the pod wall show a differentiation in the distribution of the phenolic acids and flavonoids in transverse section. The amounts of individual phenols present, moreover, vary in relation to the degree of maturity of the tissue (Tables 1 and 2). Only trace amounts of the phenolic acids were detected in the hydrolysates of bean tissues from mature pods, and immature beans excised from developing pods were found to contain none of the phenolic acids detected in other cacao tissues.

Leaf. The mature green leaf was shown to contain a high level of compounds of the cinnamic acid group. These acids, with the exception of caffeic acid, are present only in traces in the young leaf, which contains appreciable amounts of quercetin. During the period in which the red pigmentation of the leaf is lost and succeeded by the characteristic green of the mature leaf, a gradual loss of quercetin accompanied by an increase in the amount of caffeic, *p*-coumaric, sinapic and ferulic acid is apparent.

In order to explore the possibility that some of the phenolic acids detected in leaf hydrolysates might occur naturally in the free state, methanolic extracts prepared by the standard procedure were examined in solvent *A*. In all cases, however, it was observed that no mobile fluorescent compounds were present. On submitting the extracts to a hydrolytic treatment with 20% HCl, mobile fluorescent materials made their appearance and were identified as the phenolic acids described above. It is therefore evident that all of the phenolic acids occurring in the cacao leaf exist in the bound form, possibly as depsides. In several tissue hydrolysates, fluorescent materials of very low R_F were detected, but in view of the difficulty of obtaining sufficiently exact measurements of the R_F values to characterize these compounds, the substances were not identified.

Anthocyanidins of tissue hydrolysates. All the tissue hydrolysates examined were red in colour, to a varying degree, owing to the liberation of anthocyanidins from anthocyanins and leucocyanidins. Chromatographic examination revealed that cyanidin hydrochloride was the only anthocyanidin present (Table 1).

Methanol-soluble polyphenols of fresh tissue. The distribution of methanol-soluble polyphenols throughout the tissues of *T. cacao* is shown in Table 2. (−)-Epicatechin and leucocyanidins L_1 and L_2 were found in most tissues examined.

Leaf. A two-dimensional chromatogram of the polyphenols of the leaf shows most of the major polyphenols of *T. cacao* (Fig. 1).

The young leaf of *T. cacao*, I.C.S. 1, is characterized by an intense red coloration, which decreases during development and is succeeded by an olive–green colour and finally by the deep green of the

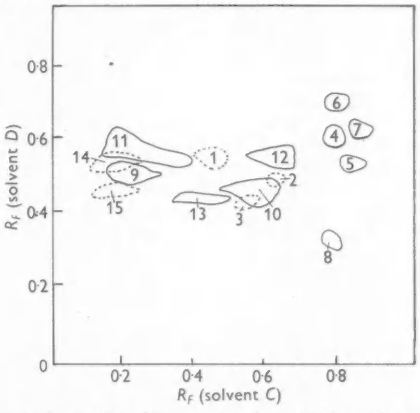

Fig. 1. Composite diagram showing position of polyphenolic components of leaf tissue. 1, (−)-Epicatechin; 2, leucocyanidin (L_1); 3, leucocyanidin (L_2); 4, chlorogenic acid; 5, neochlorogenic acid (?); 6, *p*-coumarylquinic acid; 7, compound showing violet fluorescence; 8, compound showing deep-blue fluorescence; 9, 10, 11, 12, unidentified anthoxanthins of leaf; 13, rutin; 14, 3-α-L-arabinosidylcyanidin; 15, 3-β-D-galactosidylcyanidin. Spots shown diagrammatically with a complete outline were detected by u.v. fluorescence; spots shown with a dotted outline were detected either by their colour in visible light (spots 14 and 15) or by the use of spray reagents (spots 1, 2 and 3) as described in the text.

Table 1. *Distribution of phenolic and flavonoid residues in hydrolysates of mature tissues of* Theobroma cacao

R_F values recorded (with the exception of that of cyanidin) relate to development with solvent *A* at 20°. Fluorescence in NH_3: b, blue; bg, blue–green; br, brown; g, green; v, violet; y, yellow.

	Unknown (g)	Unknown (g)	Unknown (v)	Unknown (g)	Quercetin (y)	Unknown (g)	Caffeic acid (bg)	Kaempferol (y)	Unknown (b)	Unknown (b)	*p*-Coumaric acid (v)	Sinapic acid (g)	Unknown (g)	Ferulic acid (b)	Unknown (v)	Cyanidin hydrochloride
R_F	0	0·02	0·04	0·07	0·08	0·10	0·22	0·25	0·28†	0·55	0·63	0·83	0·88	0·90	0·95	0·50*
Sap wood	–	–	–	–	+	–	–	–	+ +	–	–	–	–	–	+	+ +
Heart wood	–	–	–	–	–	–	–	–	+ +	–	–	–	+ + +	–	–	+
Bark	–	–	–	–	–	–	–	–	+ +	–	–	–	–	–	–	+ +
Root	–	+	–	–	–	–	+ + +	–	+	–	–	–	–	–	–	+
Green stem	–	–	–	–	–	–	+ +	–	+	–	+ +	–	–	–	–	+ + +
Leaf {Young	+ +	–	+ +	–	+ +	–	+ + +	+	+ +	–	+	–	–	–	–	+ + + +
Leaf {Mature	–	+ +	–	+ +	+	–	+ + +	–	+ + +	+ +	+ + +	+ +	–	+ +	+ +	+ +
Flower	–	+ +	–	–	+ + +	+ +	–	–	+	–	+ +	–	–	–	–	+ + +
Pod wall {Young {Surface	–	–	–	–	+ + +	+	+	–	+ +	–	+ +	–	–	–	–	+ + +
Pod wall {Young {Inner	–	–	–	–	+	–	+ + +	–	+	–	+ + +	–	–	–	–	+ +
Pod wall {Old {Surface	–	–	–	–	+	+	+	–	+ +	–	–	–	–	+ +	–	+ +
Pod wall {Old {Inner	–	–	–	–	+	–	+ +	–	+	–	+	–	–	–	–	+ +
Bean {Cotyledons	–	–	+	–	+	+	+ +	–	+	–	+	–	–	–	–	+ + + +
Bean {Skin and adhering tissues	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+

* R_F relates to solvent *B*.

† *Note added in proof.* This compound has now been shown to possess identical chromatographic and fluorescent properties to gentisic acid (2:5-dihydroxybenzoic acid.)

Table 2. *Methanol-soluble polyphenols of* Theobroma cacao

Fluorescence: symbols are as identified in Table 1.

	Leucocyanidin L_2	Rutin* (br)	3β-D-Galactosidyl cyanidin	Neochlorogenic acid (?)	Leucocyanidin L_1	3-α-L-Arabinosidyl cyanidin*	Chlorogenic* acid	Epicatechin*	*p*-Coumaroylquinic acid*	Unknown (bg) 0·82	Isochlorogenic acid	Unknown (g) 0·90	Unknown (v) 0·93
Sap wood	+ +	–	–	–	+ +	–	+	+	–	–	–	–	–
Heart wood	+	–	–	–	+ +	–	–	+ +	–	–	–	–	–
Bark	+	–	–	–	+ +	–	–	+ +	–	–	–	–	–
Root	+	–	–	–	+ +	–	–	+	–	+	–	–	–
Green stem	+ +	–	–	–	+ +	–	+	+ +	–	–	–	–	–
Leaf {Young	+ +	+ +	+	–	+ +	+ + +	+ + +	+ + +	+ + +	–	–	–	–
Leaf {Mature	+	+ +	–	+ + +	+	–	+	+	+	–	–	–	–
Flower	+ +	–	+ +	–	+ +	+ +	–	+ + +	–	–	–	–	–
Pod wall {Young {Surface	+ + +	–	+	–	+ + +	+ + +	–	+ + + +	–	+	–	–	–
Pod wall {Young {Inner	+ +	–	–	–	+ +	–	–	+	–	+ + + +	–	+ +	+ +
Pod wall {Old {Surface	+ +	–	+	–	+ +	+	–	+ +	–	–	–	–	–
Pod wall {Old {Inner	+	–	–	–	+	–	–	+	–	+	–	–	–
Bean {Cotyledons	+ +	–	+ + + +	–	+ +	+ + + +	–	+ + + +	–	–	–	–	–
Bean {Skin and adhering tissues	–	–	–	–	–	–	–	+	+	–	–	–	–

* Confirmed by cochromatography.

mature leaf. The red coloration was shown to be due to the presence of 3-α-L-arabinosidylcyanidin and 3-β-D-galactosidylcyanidin.

The presence of chlorogenic acid and *p*-coumarylquinic acid was observed in the young leaf (Table 2). Both of these compounds decreased in amount during growth and disappeared at maturity. During these changes two new compounds of higher R_F in solvent *D* appeared on the chromatograms, possessing a blue–green and a violet fluorescence respectively. From the position of the former compound on the chromatogram in relation to the other substances of the chlorogenic acid group, it appears probable that this substance is neochlorogenic acid. It was not, however, possible to confirm this provisional identification, as neochlorogenic acid was not available to us for purposes of chromatographic comparison. Since the rate of increase of the substance resembling neochlorogenic acid paralleled the rate of disappearance of the chlorogenic acid, a possible conversion of the former into the latter during growth is suggested.

(−)-Epicatechin, mobile leucocyanidins (Table 2) and stationary leucocyanidin material were also detected. At least five anthoxanthins, of which one was shown to be identical with rutin, are present in the leaf of cacao. The remaining anthoxanthins were not identified, the work being handicapped by the unavailability of the less-common anthoxanthins as markers.

Fruit. The major polyphenols of the pod wall were (−)-epicatechin leucocyanidins L_1 and L_2 and a fluorescent polyphenol of R_F 0·82. The latter compound, which appeared blue in u.v.-light radiation but became greener on exposure to NH_3 fumes, resembled a member of the chlorogenic acid group, but on chromatographic comparison with authentic specimens of chlorogenic and isochlorogenic acid it was found to possess an R_F value in solvent *D* intermediate between those observed for these two compounds (Table 3). The material was subsequently shown to possess an R_F value closely resembling that of caffeic acid, and the fluorescence also was observed to be very similar to that displayed by this compound. That the material is not caffeic acid, however, was shown by comparison in solvent *A*, when the pod-wall compound was found to be immobile whereas the caffeic acid showed an R_F value of 0·22 at 20°.

On submitting the fluorescent pod-wall polyphenol to acid hydrolysis, caffeic acid was detected among the hydrolysis products. Attempts to establish the presence of quinic acid with the sodium metaperiodate, sodium nitroprusside and piperazine reagents of Cartwright & Roberts (1955) were unsuccessful but this may have been due to the small amount of material available.

On spraying with diazotized *p*-nitroaniline the fluorescent compound gave rise to a purple–brown coloration, whereas spraying the chromatogram with an aqueous suspension of polyphenol oxidase, by the technique of Griffiths (1957*b*), gave rise to rapid darkening in the position of this compound.

The young pod wall of the clone, I.C.S. 1, is characterized by an intense-red pigmentation, which decreases as the pod matures. The pigments responsible for this coloration were shown to be 3-α-L-arabinosidylcyanidin and 3-β-D-galactosidylcyanidin.

A violet fluorescent material of R_F 0·93 in solvent *D*, occurring in young-pod-wall tissue, is probably a depside of *p*-coumaric acid, as this acid was detected among the products of acid hydrolysis when the compound was eluted with water and treated with 2N-HCl. It is not, however, identical with the *p*-coumarylquinic acid isolated from apple and pear tissue by Cartwright & Roberts (1955), since the *p*-coumaryl derivative of the pod wall possesses a higher R_F value and separates from admixture with an authentic specimen on development with solvent *D*.

Beans. The polyphenols of the ripe bean have previously been described by Forsyth (1952, 1955). In addition to those reported by him, evidence was obtained by two-dimensional chromatography of the presence in trace quantity of *p*-coumarylquinic acid, which was detected by its violet fluorescence in u.v.-light radiation. A fluorescent compound possessing a similar R_F value to the chlorogenic

Table 3. *Comparison of fluorescent polyphenols of pod wall and phenolic compounds showing similar fluorescent or chromatographic properties*

	R_F in solvent *D* (29°)	R_F in solvent *A* (20°)	Fluorescence in the presence of NH_3
Major fluorescent polyphenol of pod wall	0·82	0	Blue–green
Minor green fluorescent polyphenol	0·90	.	Blue–green
Minor violet fluorescent polyphenol	0·93	.	Violet
Chlorogenic acid	0·73	.	Green
Isochlorogenic acid	0·85	.	Green
Caffeic acid	0·83	0·22	Blue–green
p-Coumarylquinic acid	0·79	.	Violet

acid was detected but it differs from the latter in not showing the same degree of greening with NH_3 fumes.

During maturation of the fruit the white ovules enlarge and give rise to the heavily pigmented beans, and the tissue surrounding the ovules gives rise to the semi-fluid pulp. Samples were taken from the composite tissues internal to the pod wall, and the development of the polyphenols was followed chromatographically. Examination of the chromatograms revealed that large amounts of the leucocyanidin material, stationary in solvent *D*, were present throughout development but that the mobile leucocyanidins and (−)-epicatechin first appeared at the stage at which the anthocyanins make their appearance. Of the two anthocyanins, the 3-α-L-arabinosidylcyanidin was the first formed. The fluorescent material resembling chlorogenic acid appeared at a relatively late stage in the ripening of the bean.

Flower. The pigments present were identified as 3-α-L-arabinosidylcyanidin and 3-β-D-galactosidylcyanidin.

A large amount of (−)-epicatechin and leucocyanidins L_1 and L_2 were present.

Root tissue. Among the compounds detected were small amounts of the major fluorescent polyphenol observed in pod wall, the R_F values, fluorescence and colour reaction with *p*-nitroaniline being identical.

DISCUSSION

From the observations recorded, it is evident that in *T. cacao*, I.C.S. 1, the phenolic compounds vary not only with the morphology of the tissue but also with the physiological age of the material.

Of the phenolic acids detected in hydrolysates, caffeic and *p*-coumaric acid are known to arise at least in part from chlorogenic acid and *p*-coumarylquinic acid respectively, whose presence has been established. Evidence was also obtained for the presence of bound forms of ferulic and sinapic acid in the leaf but the nature of the parent substances remains unknown.

Young-leaf tissue is characterized by the presence of large amounts of the leucocyanidins, which show a progressive decrease during growth. A similar relationship between the age of the leaf and the leucocyanidin content of *Eucalyptus sieberiana* has been noted by Hillis (1956). The metabolic fate of the leucocyanidin in the developing leaf of *T. cacao* is uncertain but the parallel increase in the phenolic acid content is suggestive. It has recently been shown by the use of radioactive tracers that certain of the aromatic acids, namely cinnamic and *p*-hydroxycinnamic acid, were very good precursors of quercetin in buckwheat (Underhill, Watkin & Neish, 1957), and it appears not unlikely that the biosynthetic route is reversible. Whether or not the more complex polyphenols of young cacao-leaf tissues are degraded to the phenolic acids detected in mature leaf tissue is a question which could be settled conclusively only by the use of labelled compounds.

In the course of this study consideration was given to the possible origin of the phenolic substances of the bean. It would appear equally possible that the bean polyphenols are synthesized completely in the cotyledon or that the aromatic ring or flavonoid skeleton is elaborated in the leaf and translocated to the developing ovule. In support of the latter view Lindner, Fitzpatrick & Weeks (1950) found that any type of phloem blockage produced accumulation of polyphenol, and from their description it appears that these polyphenols could have been leucocyanidins. The view that the bean polyphenols may arise from preformed leucocyanidin is also supported by the observation that the simpler hydroxycinnamic acids, which have been recognized as early precursors of the flavonoid skeleton (Underhill *et al.* 1957), were absent from the developing bean although present as depsides in the mature leaf. The possibility must be recognized also of formation of the flavonoids of the ripe bean from the immobile leucocyanidin material, which is present before and throughout the period during which the anthocyanins, catechins and mobile leucocyanidins make their appearance in the developing bean. That the leucocyanidins of certain plants may be the precursors of anthocyanin pigments has previously been suggested by Robinson & Robinson (1939), Simmonds (1954) and Alston & Hagan (1955). That the leucocyanidins are present in large amounts in rapidly growing leaf tissue and low in the metabolically inert tissues, e.g. bark and heart wood, suggests that in *T. cacao* the leucocyanidins play an active role in the intermediary metabolism of the plant and are not to be regarded as inert end-products.

It has also been noted that large amounts of leucocyanidin are always found in tissues containing appreciable quantities of the anthocyanins, the young leaf, the bean and the flower being particularly rich in both types of compound. The converse, that colourless tissues are necessarily low in leucocyanidins, does not, however, hold since the young unpigmented inner-pod-wall tissue contains appreciable amounts of leucocyanidin material.

SUMMARY

1. The distribution of phenolic compounds in the tissues of *Theobroma cacao* is described.

2. (−)-Epicatechin and leucocyanidins L_1 and L_2 were present to a varying degree in all tissues.

3. Caffeic acid, *p*-coumaric acid and an unidentified fluorescent compound of R_F 0·28 are present in many cacao tissues, but ferulic and inapic acids are of limited occurrence.

4. The major anthocyanin of the young leaf and young pod wall was shown to be 3-α-L-arabinosidylcyanidin, and in the flower 3-α-L-arabinosidylcyanidin and 3-β-D-galactosidylcyanidin were present in approximately equal amounts.

5. Cyanidin hydrochloride was the only anthocyanidin detected in tissue hydrolysates of *T. cacao*.

6. *p*-Coumarylquinic acid was detected in the cotyledons of the bean and in young-leaf tissue. Chlorogenic acid was also shown to occur in young-leaf tissue.

7. A fluorescent phenolic compound releasing caffeic acid on hydrolysis, but not identical with chlorogenic or isochlorogenic acid, was detected in pod-wall tissue.

8. Leucocyanidin material is present in large amounts in young-leaf tissue but the content falls to a very low level at maturity. Similar developmental changes were observed in pod-wall tissue.

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Purification of Free Staphylococcal Coagulase

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Two types of material capable of interacting with plasma or fibrinogen are formed by pathogenic staphylococci. One of these, termed bound coagulase, is apparently part of the cell wall and is released only by disintegration or autolysis, whereas the second, known as free coagulase, is liberated into the medium during growth (Duthie, 1954*a*). Free coagulase has been shown by numerous workers to interact with a so-called coagulase activator found in the plasma of certain animals to give a thrombin-like material capable of clotting fibrinogen (Smith & Hale, 1944). The nature of the coagulase activator is disputed, but recently the work of Tager (1956) suggests that both prothrombin and another factor, possibly a prothrombin-breakdown product, can function in this respect. The nature of their interaction is unknown.

Partial purification of free coagulase from cultures in digest broth was described by Duthie & Lorenz (1952). They found that coagulase was easily precipitated by cadmium salts, which could be removed by dialysis at an acid pH, giving a coagulase which was relatively stable in solution at pH 2·0. Jacherts (1956) has recently submitted material prepared in this way to further purification by column chromatography on aluminium hydroxide, followed by paper electrophoresis. A single active component staining with ninhydrin was obtained, but no details regarding its specific activity are given.

In the present purification studies, use was made of the finding by Duthie (1954*b*) that if 1 volume of a fully grown shaken culture of staphylococci in digest broth is inoculated into 9 vol. of fresh warm broth, i.e. to give a concentration of about 40 μg. of bacterial nitrogen/ml., and is then shaken at 37°, there is a maximal release of free coagulase in less than 2 hr. By inoculating into a casein-hydrolysate medium instead of digest broth and growing for exactly 1 hr. 20 min. a maximal ratio of free

coagulase to extracellular protein was obtained. This relatively pure starting material was then concentrated by the cadmium-precipitation method already described. The material was stable only if precipitated by ammonium sulphate and stored as a dry powder. By fractionation with ammonium sulphate at low pH values, it was finally possible to obtain coagulase in an electrophoretically pure form. The stages in this purification are the subject of this paper.

MATERIALS AND METHODS

Culture medium. (*a*) The digest broth was Hartley's broth prepared from ox heart (Mackie & McCartney, 1956). Equally good results were obtained with Bacto Brain–Heart Infusion Broth (Difco Laboratories, Detroit, Mich., U.S.A.). (*b*) The casamino acid medium used was similar to that given by Rogers (1945) as medium C, with the following modifications: (1) the casamino acid (Oxoid; Oxo Ltd., London) content was 2% (w/v); (2) hydrated sodium β-glycerophosphate was 1·25% (w/v); (3) the final pH after seeding was 7·4.

The final medium was made as follows and it is referred to as CAA medium. Casein acid hydrolysate (Oxoid), 500 g., anhydrous Na_2HPO_4, 70 g. and KH_2PO_4, 100 g., were dissolved in 3 l. of water. The pH was adjusted to 9·0 with 10N-NaOH. The solution was boiled for 30 min., cooled and filtered. The filtrate was made approximately neutral with 10N-HCl. Tryptophan, 0·5 g., cystine, 2·5 g., and tyrosine, 2·5 g., were added in solution. A stock salt solution containing anhydrous $CuSO_4$, $ZnSO_4$ and $FeSO_4$, each at 0·5% dissolved in N-HCl, was prepared and 0·5 ml. of this was added.

The volume was adjusted to 5 l. with water, and the pH to 6·8 with 10N-HCl. This concentrated medium was dispensed in 200 ml., bottles and autoclaved at 20 lb./in.² pressure for 20 min. Solutions containing sodium β-glycerophosphate, 50% (w/v), and glucose, 5% (w/v), were dispensed in 100 ml. volumes and steamed for 15 min.

When required, 200 ml. of the concentrated acid hydrolysate as above, 25 ml. of the sodium β-glycerophosphate–glucose solution, 0·2 ml. of 0·1% thiamine and 2 ml. of 0·1% nicotinamide were made up to 1 l. with non-sterile water immediately before use. The final pH was 7·6.

Shaking. Penicillin flasks containing 550 ml. of culture were shaken on a horizontal table made to rotate eccentrically about 56 times/min. with a horizontal throw of 12 cm.

Organism used. The Newman strain of *Staphylococcus aureus* (Duthie & Lorenz, 1952), N.C.T.C. 8178, was used.

Filters. Hyflo Super-Cel (Johns Manville, 22 East 40th Street, New York 16, U.S.A.), 16 g., was boiled for 1–2 min. with 200 ml. of 2N-NaOH. This was cooled and filtered through two Whatman no. 1 filter papers in a 15 cm. Büchner funnel. A third filter paper was placed over the earth and the whole pad was washed with 4–5 l. of water. A filter prepared in this way removed bacteria from 20 l. of lightly centrifuged culture supernatant with minimal loss of coagulase.

Assay of coagulase. Fresh human plasma, diluted 1 in 5 with saline, was added in 0·2 ml. amounts to tubes measuring 5·0 cm. × 0·8 cm. in a water bath at 37°. Immediately before use one drop (0·02 ml.) of 6% (w/v) Filter-Cel (Johns Manville) was added to each tube. A volume (0·2 ml.) of coagulase, diluted if necessary in 0·067M-Na_2HPO_4–KH_2PO_4 Sørensen buffer, pH 6·8 (Clark, 1928), was added to a tube and viewed by indirect light against a black background, the tube being slowly tapped with one finger. The end-point was recognized by a rapid agglutination of the Filter-Cel particles. A curve was constructed for dilutions of coagulase, giving clotting times between 15 and 40 sec. A solution which, when diluted one part in two in buffer and tested as above, gave a clotting time of 20 sec. was defined as containing 1 unit/ml. Approximate values for concentrations giving longer clotting times than 40 sec. were obtained by extrapolating the straight line obtained when clotting time was plotted against the reciprocal of the coagulase concentration. Fig. 1 shows that the curve for culture supernatants differed from that for purified material. One unit as above equalled approx. 3500 minimal clotting doses as defined previously (Duthie & Lorenz, 1952). All preparations of coagulase were spun free of precipitates or bacteria, and were neutralized if necessary before testing.

Fibrinogen plates. These contained approx. 75 mg. of human or bovine fibrinogen/100 ml. and 0·2% (v/v) of human plasma (Klemperer & Haughton, 1957). Human fibrinogen was obtained from the Blood Products Laboratory, Lister Institute, Elstree, Herts, and bovine fibrinogen was bovine-plasma fraction I obtained from Armour Ltd., Hampden Park, Eastbourne, Sussex.

Culture storage. Strains were stored after drying by the method of Stamp (1947).

Growth measurement. This was expressed as μg. of bacterial nitrogen/ml. and measured by the indirect method of Rogers (1954) with a Spekker photo-electric colorimeter and spectrum-red filters. Triplicate samples were taken from a shaken culture of *S. aureus* strain Newman growing in

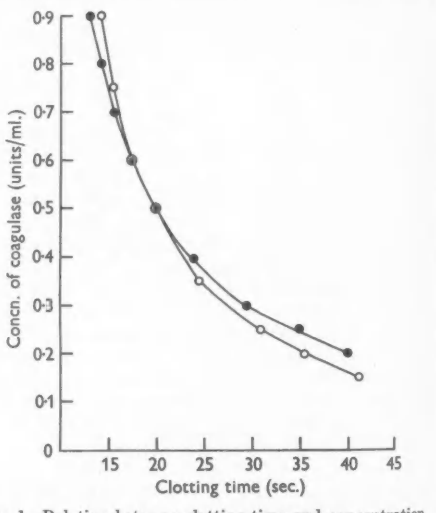


Fig. 1. Relation between clotting time and concentration of free coagulase; in culture supernatants (○) and after extraction from culture supernatant (●).

CAA at 1 and 3 hr. after inoculation with 10% (v/v) of a digest-broth culture shaken for 24 hr. Sample 1 was suitably diluted with CAA and the opacity of a range of dilutions measured. Duplicate samples 2 and 3 were centrifuged, and the cells washed twice with saline and total nitrogen measured by a standard micro-Kjeldahl technique. It was found that the relationship between opacity and bacterial nitrogen was the same for both sets of samples.

Protein. Soluble extracellular protein in culture fluid was measured, by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine albumin (Armour Ltd.) as a standard. In the purification process (Table 5), total protein was calculated from the absorption at 260 and 280 mμ as measured with a Unicam SP. 500 spectrophotometer, with the formula of Kalckar (1947).

Measurement of pH. pH was measured on a Pye Master pH meter.

RESULTS

Early experiments suggested that a main reason for low coagulase production was the appearance of non-coagulase-producing variants which rapidly outgrew coagulase-producing cells. This was tested as follows. A single coagulase-producing colony, recognized on a fibrinogen plate by the production of a large opaque halo around the colony, was inoculated into 20 ml. of 2% (w/v) Bacto-tryptone (Difco) and shaken on an inclined gramophone turntable for 24 hr. at 37°. A loopful (2 mm. diameter) of this culture was then inoculated into 20 ml. of 2% (w/v) Bacto-tryptone and shaken for a further 24 hr. Subculturing was continued in this manner for a total of five cultures. Each culture was plated on fibrinogen–agar to give discrete colonies and coagulase production assessed on the basis of halo diameter, when three distinct kinds were recognized: (*a*) large haloes about 7–9 mm. diameter; (*b*) reduced haloes about 2–4 mm. diameter; (*c*) halo absent. About 500 colonies were counted in each culture and the following was found. All of the colonies in the first and second cultures had large haloes. Of the cells in the third culture 90% gave large haloes and 10% had none. In the fourth culture the proportions were 25% large haloes, 74% without haloes and 1% reduced haloes. In the fifth culture no colonies produced large haloes, 95% were without haloes and 5% had reduced haloes. All three types had identical phage patterns. Rogers (1953) has noted a similar variation in the ability of *S. aureus* strain no. 524 to produce hyaluronidase.

In order to obtain culture supernatants containing the minimum amount of protein consistent with high concentrations of coagulase, it would have been desirable to grow both the inoculum and the inoculated culture in casein-hydrolysate medium. However, it was not possible to use casein-hydrolysate medium for growth of the inoculum, since this resulted in a culture containing a low concentration of coagulase of poor specific activity (Table 1). A similar result was obtained whether the inoculum consisted of whole culture or cells only.

When the inoculum was grown in digest broth and inoculated in casein-hydrolysate medium, coagulase in high concentration and of high specific activity was obtained whether the inoculum consisted of whole culture or cells only. Although the digest-broth culture contained approx. 500 μg. of soluble protein/ml. it was decided to use whole culture rather than cells only, for two reasons. If the inoculum consisted of cells only, then there was not only a somewhat lower yield of coagulase (Table 1) but, more important, a very substantial loss occurred when the cells were removed from the culture by filtration through Hyflo Super-Cel. Digest-broth cultures appeared to contain a substance which protected the coagulase from adsorption on the earth, and for this reason whole cultures in digest broth were used as inocula.

Shaking. All previous work on the production of staphylococcal toxins, commencing with that of Casman (1940), has shown that shaking is essential for obtaining rapid and maximal production. Since large volumes of culture medium were needed a comparison was made of some of the methods available. With a Vortex stirrer such as that used by Chain, Paladino, Callow, Ugolini & van de Sluis (1952), growth was adequate, but the coagulase yield was very low (about 0·1–0·2 unit/ml.). Most

Table 1. *Influence of the preparation of inoculum on production of coagulase by* Staphylococcus aureus *strain Newman in CAA medium*

A volume (50 ml.) of seed culture, shaken for 24 hr., grown in either digest broth or CAA (either whole culture or cells only) was added to 500 ml. of CAA and shaken in a penicillin flask at 37°. At the indicated times a 10 ml. sample was removed from each flask and immediately centrifuged. Coagulase (units) and soluble protein precipitable by trichloroacetic acid were measured in the supernatant.

Growth time (hr.)	Seed grown in digest broth and whole culture used		Seed grown in digest broth and cells only added		Seed grown in CAA and cells only added	
	(units/ml.)	(units/mg. of protein)	(units/ml.)	(units/mg. of protein)	(units/ml.)	(units/mg. of protein)
1	1·2	19·0	0·70	21·4	0·12	6·0
1·5	1·35	13·5	1·0	20·8	0·2	5·7

successful was either of the following: (i) culture in penicillin flasks (cylindrical flasks 17 cm. diameter and 10 cm. deep) shaken on the horizontal table described above, or (ii) 20 l. bottles containing some 4 l. of culture and rolled in the horizontal position. Since the former were easier to handle and were available, they were used in all the experiments.

Initial culture density and time of growth. That the inoculation of 1 vol. of 24 hr. shaken digest-broth culture into 9 vol. of fresh casein-hydrolysate medium (i.e. 10%, v/v) gave the highest yield consistent with greatest specific activity is clear from Table 2, which is representative of several experiments giving similar results. A 20% (v/v) inoculation gave a slightly higher yield with lower specific activity, and 4% (v/v) gave a similar specific activity and lower yield. If, as in Fig. 2, the casein-hydrolysate medium was used with a 10% (v/v) inoculation then the highest specific activity was found between 80 and 90 min.

Bacterial growth, total extracellular protein precipitable by trichloroacetic acid and coagulase were measured at 20 min. intervals in five cultures grown under apparently identical conditions on 5 successive days. Maximum coagulase concentrations in the five cultures varied from 1·6 to 2·1 units/ml. and maximum specific activities varied

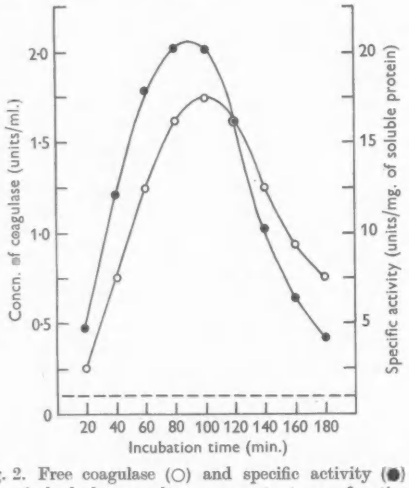


Fig. 2. Free coagulase (○) and specific activity (●) in casein-hydrolysate-culture supernatants as a function of incubation time. The broken horizontal line represents the inoculum level of coagulase.

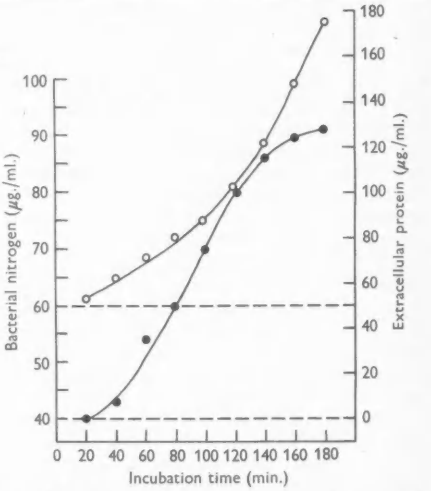


Fig. 3. Bacterial growth (●) and extracellular protein precipitable by trichloroacetic acid (○) in a casein-hydrolysate culture as a function of incubation time. The broken horizontal lines represent inoculum levels of bacterial nitrogen and extracellular protein.

Table 2. *Effect of initial culture density and length of incubation period on final yield and specific activity of coagulase liberated by* Staphylococcus aureus *strain Newman in CAA medium*

A digest-broth culture, shaken for 24 hr., containing 0·8 unit of coagulase/ml. and 500 μg. of soluble protein precipitable by trichloroacetic acid/ml. was diluted with CAA medium previously warmed to 37°. The volume of digest-broth culture indicated was diluted to give a final volume of 550 ml. and shaken at 37° in a penicillin flask. At the times stated a 10 ml. sample was removed from each flask and immediately centrifuged. Coagulase (units) and protein precipitable by trichloroacetic acid were measured in the supernatants.

	Coagulase concentration and specific activity in culture supernatants							
	Vol. (ml.) of digest-broth seed present/550 ml. of culture							
	275		110		55		22	
Growth time (hr.)	(units/ml.)	(units/mg. of protein)	(units/ml.)	(units/mg. of protein)	(units/ml.)	(units/mg. of protein)	(units/ml.)	(units/mg. of protein)
1	0·5	1·7	1·35	11	1·15	20	0·5	20
1·5	0·5	1·7	1·25	8	1·25	18	0·7	19
2	0·4	1·6	1·25	7	1·25	14	1·0	18
3	0·4	1·2	1·25	6·3	1·25	10	1·0	14

Table 3. *Effect of volume of culture grown in each penicillin flask on production of coagulase by* Staphylococcus aureus *strain Newman*

A volume (200 ml.) of a digest-broth culture shaken for 24 hr. was seeded into 2 l. of CAA medium previously warmed to 37°. The volume indicated was shaken in a penicillin flask at 37° for 80 min. Coagulase measurements were then made on culture supernatants.

Culture volume (ml.)	Concn. of coagulase (units/ml.)	Total units of coagulase produced
50	2·0	100
100	2·9	290
200	2·5	500
350	2·3	800
550	2·1	1160
750	1·5	1125

from 19 to 26 units/mg. of extracellular protein precipitable by trichloroacetic acid. Results for bacterial growth and extracellular protein precipitable by trichloroacetic acid were reproducible to within 10%. When each of these functions was plotted against time, curves of similar shape were obtained in each experiment. In all cases the highest concentration of coagulase was reached between 80 and 100 min. after inoculation and maximum specific activity between 70 and 90 min. after inoculation. The results obtained from one of these cultures are presented in Figs. 2 and 3. It should be noted that the liberation of coagulase occurred before the termination of rapid multiplication of the bacteria, and before the liberation of extracellular protein had reached its highest rate. The final conclusion drawn was that an inoculum of 10% (v/v) of a digest-broth culture, shaken for 24 hr. into casein-hydrolysate medium, giving a final concentration of 40 μg. of bacterial nitrogen/ml. when shaken for 80 min. in penicillin flasks, gave greatest yield of coagulase consistent with greatest specific activity.

Culture volume. Experiments were carried out to determine the greatest volume of culture, consistent with high yields of coagulase, which could be grown in a penicillin flask. The results of one such experiment are given in Table 3. In all cases about 500 ml. proved to be the maximum amount in which high concentrations of coagulase were obtained. When less than 500 ml. of culture was grown in each flask, higher concentrations of coagulase were obtained, but the total amount of coagulase was less.

Constituents and pH *of medium.* Each of the constituents of the casein-hydrolysate medium given under Methods was varied independently and the effect of this variation on coagulase production noted. The results, which are typical of several experiments, are given in Table 4. The minimum concentration of each constituent giving highest coagulase production was as follows: (i) casein hydrolysate, 2% (w/v); (ii) hydrated sodium β-glycerophosphate, 0·625% (w/v); (iii) glucose, 0·025% (w/v). These values did not vary in the different experiments.

It had previously been noticed that inoculation of the casein-hydrolysate medium with 0·1 vol. of a digest-broth culture shaken for 24 hr. reduced the pH from 7·6 to 7·4, and that after growth for

Table 4. *Effect of independent variation of concentration of casein hydrolysate, sodium β-glycerophosphate and glucose, and of initial* pH *of seeded culture on production of coagulase by* Staphylococcus aureus *strain Newman in CAA medium*

A volume (50 ml.) of digest-broth culture shaken for 24 hr. was seeded into 500 ml. of test medium previously warmed to 37°. The cultures were shaken in penicillin flasks for 80 min. at 37°. Samples were centrifuged immediately and all coagulase measurements were made on the cell-free supernatants. pH measurements were made on whole cultures.

(*a*) Varying concentration of casein hydrolysate

Concn. of casein hydrolysate (%, w/v)	Concn. of coagulase (units/ml.)
5	1·6
4	2·0
2	2·0
1	1·7
0·5	1·2

(*b*) Varying concentration of sodium β-glycerophosphate

Concn. of sodium β-glycerophosphate (%, w/v)	pH after growth for 80 min.	Concn. of coagulase (units/ml.)
3·75	7·0	1·6
1·25	6·8	2·2
0·625	6·8	2·2
0·25	6·8	1·7
Nil	6·75	1·8

(*c*) Varying concentration of glucose

Concn. of glucose (%, w/v)	Concn. of coagulase (units/ml.)
0·5	1·9
0·125	2·0
0·025	1·9
0·0125	1·5
Nil	1·2

(*d*) Varying initial pH

Initial pH of seeded culture	pH after growth for 80 min.	Concn. of coagulase (units/ml.)
6·50	6·40	1·0
6·85	6·65	1·4
7·10	6·90	1·8
7·40	7·15	1·9
7·55	7·35	2·0
7·70	7·40	1·8

80 min. the pH had fallen to between 6·8 and 7·2. It was therefore thought possible that the influence of sodium β-glycerophosphate might be due to buffering of the culture and that coagulase production might be influenced by pH alone. The point was investigated independently and it was found, provided that the initial pH of the inoculated culture was between 7·1 and 7·7, that there was no significant difference in the concentration of coagulase produced (Table 4). Furthermore it was found that the pH of the culture after growth for 80 min. was identical at concentrations of sodium β-glycerophosphate from 0·25 to 1·25 % (w/v).

In addition, the supplementary amino acids recommended by Rogers (1945) were tested and it was found that the addition of tyrosine (100 mg./l.) and tryptophan (20 mg./l.) to the basic medium either singly or together caused approx. 50 % increase in coagulase production. The addition of cystine (100 mg./l.) was without effect on the final coagulase content of the culture, but this point was not investigated until relatively late in the work and cystine was always included in the medium.

Removal of cells. Since delay in the removal of the cells from cultures grown for 80 min. under the conditions finally used resulted in the further liberation of soluble protein, it was important that the cells should be removed as quickly as possible. The most convenient and rapid method was by centrifuging for 7 min. at 3000 *g* followed by Büchner-funnel filtration with Hyflo Super-Cel.

In choosing Hyflo Super-Cel the following experiment was done. Filter pads were made from 2 g. of each of the following earths: Hyflo Super-Cel, Celite 535, Filter-Cel (all obtained from Johns Manville Co.), kieselguhr B.P. (acid-washed) and kaolin light B.P. (obtained from British Drug Houses Ltd., Poole, Dorset). When 200 ml. volumes of neutral, lightly spun cultures in casein-hydrolysate medium were filtered through the pads, some adsorbed the coagulase almost completely, whereas others failed to retain the bacteria. Good retention with low loss of coagulase was found in Hyflo Super-Cel pads, particularly if the earth was treated by boiling with 2N-NaOH for 1–2 min. It was very important that the same Hyflo Super-Cel pad should be used for as large a volume as possible in order to reduce the loss of coagulase due to adsorption.

When the majority of the bacteria had been removed from the culture by centrifuging it was found that one Hyflo Super-Cel filter pad, prepared as described in Methods, could be used to filter 20–25 l. of culture. If prior centrifuging was omitted, large numbers of bacteria passed through the pad after 2–3 l. of culture had been filtered. Small batches were cleared of bacteria by prolonged centrifuging without recourse to filtration.

Purification of coagulase

Stage I. Cadmium sulphate (670 g.) dissolved in about 2 l. of warm water was stirred into 246 l. of cell-free-culture supernatant containing coagulase at a concentration of 1·7 units/ml. The flocculent precipitate, which contained more than 90 % of the coagulase, was allowed to settle at 4° for 16 hr. Most of the supernatant fluid was decanted and the remainder removed by centrifuging. The precipitate was washed twice with cold water and resuspended in water as a thick slurry. 2N-HCl was added very slowly, with constant stirring at 4°, until a clear solution was obtained and the pH was 2·0. This solution was then dialysed for 48 hr. against a constant flow of KCl–HCl buffer, pH 2·0, *I* 0·1.

Stage II. Ammonium sulphate crystals were added to the 7825 ml. of solution at 4° to give a final concentration of 0·67 saturation. The precipitate was allowed to form for 1 hr., collected by centrifuging and dialysed at pH 2·0 for 24 hr. A certain amount of insoluble material was centrifuged down and discarded. The 594 ml. of supernatant contained 53 % of the coagulase at a concentration of 360 units/ml.

Stage III. The coagulase was reprecipitated by the addition of 1188 ml. of a saturated solution of $(NH_4)_2SO_4$ previously adjusted to pH 2·0 with 10N-HCl. After standing at 4° for 1 hr. the precipitate was collected by centrifuging, washed with 0·67 saturated $(NH_4)_2SO_4$ and dried *in vacuo* over P_2O_5. The yield was 27 g. of dried powder, containing 46 % of the original coagulase activity.

Stage IV. The powder was resuspended and dialysed against a constant flow of buffer, pH 2·0, for 48 hr., then adjusted to pH 2·7 and fractionally precipitated by the slow addition of a saturated solution of $(NH_4)_2SO_4$ previously adjusted to pH 2·7 with 10N-HCl. It was found that 65 % of the coagulase was precipitated between 0·10 and 0·20 saturation. Approximately a fourfold purification was achieved by this step. The precipitate was collected and dialysed against buffer, pH 2·0, for 24 hr. at 4°.

Stage V. This solution (49 ml.) was refractionated with $(NH_4)_2SO_4$ at pH 2·0. The precipitate which formed between 0·125 and 0·175 saturation, although containing only about 20 % of the activity, showed greatly enhanced specific activity.

Stage VI. On dialysis against Na_2HPO_4–citric acid buffer, pH 5·2 (McIlvaine, 1921), *I* 0·1, a precipitate formed, which was removed, leaving a solution of an electrophoretically homogeneous protein. It was not found possible to remove the remaining impurity in the fraction precipitated between 0 and 0·125 saturation, either by dialysis at pH 5·2 or by further fractionation with $(NH_4)_2SO_4$.

Table 5. *Purification of free coagulase produced by* Staphylococcus aureus *strain Newman*

For full details, see text.

Stage of purification	Vol. (ml.)	10^{-3} × Total activity (units)	Yield (% of original activity)	Specific activity (units/mg. of protein)
Culture supernatant fluid	$2{\cdot}46 \times 10^5$	410	100	25
I Precipitated by cadmium sulphate (670 g.) Cadmium removed by dialysis for 48 hr. at pH 2·0	$7{\cdot}8 \times 10^3$	—	—	—
II After pptn. in 0·67 saturated ammonium sulphate and dialysis at pH 2·0	594	215	53	—
III Reprecipitated with 0·67 saturation of ammonium sulphate: dried over P_2O_5	27·0*	190	46	64·5
IV Ammonium sulphate fractions, pH 2·7:				
(i) 0·10 saturated ppt. (dissolved)	133	38	9·5	22
(ii) 0·20 saturated ppt. (dissolved)	49	106	26	246
(iii) 0·30 saturated ppt. (dissolved)	37	8·15	2	62
V Ammonium sulphate fractionation of IV (ii), pH 2·0:				
(i) 0·125 saturated ppt. (dissolved)	24	89	21·8	326
(ii) 0·175 saturated ppt. (dissolved)	15·7	23·1	5·6	394
(iii) 0·25 saturated ppt. (dissolved)	21	4·35	1·1	168
VI Dialysed V (ii): phosphate–citrate buffer, pH 5·2, *I* 0·1	18	19·8	4·8	430

* Weight (g.).

A summary of the purification procedure is given in Table 5.

Properties of purified coagulase

Stability. The dried powder obtained in stage III of the purification was stable for several months when stored over P_2O_5 *in vacuo* at 4°. After dialysis at pH 2·0 and freezing and storage at −18° at this pH for 1 week, there was a 30 % loss of activity but there was no loss at 4° in 1 week. The corresponding losses at neutral pH were much greater, being 30 and 50 % respectively.

The purified coagulase obtained in stage V was, however, much less stable. Approx. 50 % was lost in 1 week at pH 2·0 or pH 7·0 at both −18° and 4°. No attempt was made to store the purified coagulase in $(NH_4)_2SO_4$ over P_2O_5.

Earlier attempts at freeze-drying, with an impure preparation, resulted in a high initial loss of coagulase activity, i.e. 20–40 % loss on drying. The freeze-dried product stored at 4°, either *in vacuo* or in the presence of air, continued to lose activity, and after 1 month the loss was nearly 90 %. Neither the initial loss nor the subsequent loss on storage could be prevented by the addition of any of the following: glucose, sucrose, glycine, cysteine, albumin, citrate or ethylenediaminetetra-acetic acid.

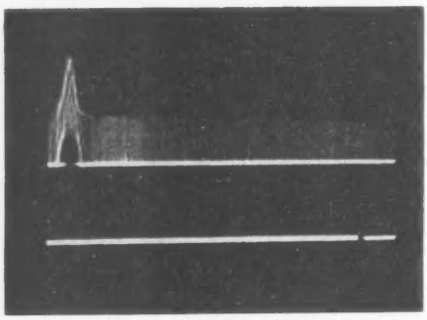
Biological properties. Purified coagulase is an extremely active clotting agent both *in vitro* and *in vivo*. As little as 75 μμg./ml. clotted human plasma in 24 hr. at 37°, and 22 μg./kg. body wt. killed rabbits by intravascular thrombosis when given intravenously. Smith & Johnstone (1956) first noted this fact. At least ten times this dose was given subcutaneously or intraperitoneally without damage. Mice possess little or no coagulase activator in their plasma and the intravenous toxic dose was some 30 times that for rabbits, being 750 μg./kg.

Purified specimens of free coagulase did not contain any of the following activities in detectable amounts: α-lysin (measured by the method of Duthie & Wylie, 1945), hyaluronidase (Rogers, 1954), penicillinase (measured by method 2 given by Duthie, 1947) or leucocidin (personal communication from Dr G. P. Gladstone).

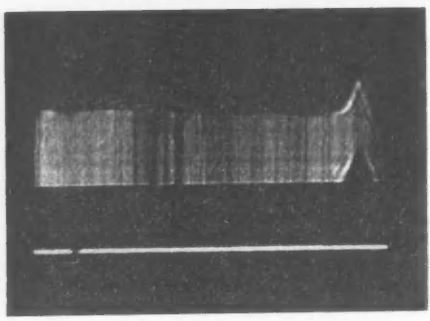
Electrophoresis. The purified coagulase was investigated in the Perkins–Elmer Tiselius apparatus at pH 3·6 (Fig. 4), 5·2 and 6·8 in Na_2HPO_4–citric acid buffers (McIlvaine, 1921), *I* approx. 0·1. Boundary analysis showed a single symmetrical peak in each case. Another preparation with a specific activity of 400 units/mg. of protein, i.e. about 90 % pure, was examined at pH 2·0 in KCl–HCl, *I* 0·1. This preparation contained two electrophoretic components. All measurements were made at 0°. The observed mobilities are presented in Fig. 5 as a function of pH and give a straight line corresponding to an approximate isoelectric point of pH 5·3. It must be stressed that this is only an approximate value since it was possible to make only one or at the most two measurements at each pH value. In order to obtain an accurate estimate

of the isoelectric point by this method it is necessary to measure mobility at several protein concentrations for each pH and extrapolate the results obtained to zero concentration, but this was not done.

Ultracentrifuging. Two samples, each having a specific activity of 400 units/mg. of protein, were examined in the Spinco model E ultracentrifuge: both preparations showed a simple asymmetrical peak. The first sample contained approx. 2% (w/v) of protein in Na_2HPO_4–citric acid buffer (McIlvaine, 1921), pH 6·8, $I \sim 0{\cdot}4$, and gave a sedimentation coefficient of 3·88. The second sample contained approx. 0·5% of protein in a buffer containing 0·05 M-KCl, 0·025 M-Na_2HPO_4 and 0·025 M-KH_2PO_4, pH 6·8, $I \sim 0{\cdot}2$, and gave a sedimentation coefficient of 4·29. From $S = 4{\cdot}29$ a very approximate minimum mean molecular weight of 44 000 can be derived. This figure could conceivably be doubled by the unknown factors.



(*a*)



(*b*)

Fig. 4. Boundary patterns obtained on electrophoresis of purified free staphylococcal coagulase at pH 3·6 in Na_2HPO_4–citric acid buffer (McIlvaine, 1921), $I \sim 0{\cdot}1$; (*a*) ascending boundary, (*b*) descending boundary.

DISCUSSION

The purification was greatly simplified by the relative purity of the starting material, which had a specific activity of 25 units/mg. of protein and was therefore about 6% pure relative to the final electrophoretically homogeneous preparation. This relative purity depended not only on the peculiar character of the Newman strain (referred to by Duthie, 1954*b* as strain 2), but also on the nice adjustment of growth conditions, so that the bulk of the coagulase was formed within a short period (80 min.), during which time the coagulase content increased from about 0·1 unit/ml. to 1·75 units/ml. and the total extracellular protein increased only from 53 to 83 μg./ml. A further point was the rapid separation of the cells from the culture medium, thereby avoiding any further liberation of non-coagulase protein from the cells.

The relationship between bacterial growth, extracellular protein and the formation of both hyaluronidase and free coagulase has been extensively studied by Rogers (1954). It is doubtful if his results are directly applicable to the present studies since he used very much smaller inocula, but he noted the absence of any lag in the production of coagulase when fully grown cells were seeded into fresh warm medium. He also found the specific rate of coagulase formation to be slower than cell growth, but he did not find any decrease in the coagulase present after growth had ceased. This is contrary to the present studies, where there was rapid loss of coagulase after 2 hr. (Fig. 2). This

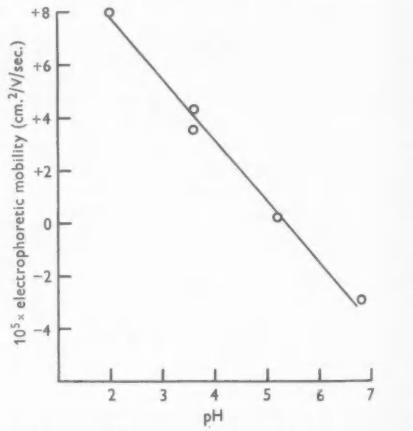


Fig. 5. Electrophoretic mobility of purified free coagulase at 0° plotted against pH. Protein strength in each ~1% (w/v). Buffers used were: KCl–HCl, pH 2·0, $I \sim 0{\cdot}1$; Na_2HPO_4–citric acid, pH 3·6, 5·2 and 6·8, $I \sim 0{\cdot}1$.

discrepancy may be due to differences in the medium used, since in our experiments growth in relatively rich media such as digest broth resulted in a decreased rate of loss of coagulase. After incubation for 16 hr. such cultures still contained about 1·0–1·5 units of coagulase/ml., compared with a maximum of about 2·0–2·5 units/ml. In the present study it was also found important to determine not only the essential constituents of the medium, but to use them in the correct concentration. Our studies on this point showed that, for the most part, Rogers's (1945) medium C was adequate for coagulase production, but not for the preparation of inoculum. In addition, casein hydrolysate was shown to contain inadequate amounts of aromatic amino acids, but the addition of cystine as in medium C was unnecessary. The addition of sodium β-glycerophosphate enhanced the production of coagulase. This was not due to its buffering capacity (Table 4*b*), but possibly because it was metabolized.

The instability of the enzyme at pH values above 2° excluded many of the adsorption techniques such as ion-exchange chromatography, since this involved working at above pH 2·0 for long periods. On the other hand, the stability of the enzyme at pH 2·0 made it possible to use such adsorbents as cadmium sulphate and aluminium phosphate, which could be dialysed away at this pH. Another important technique, that of alcohol precipitation, could not be used since coagulase together with its impurities was soluble in 95 % ethanol at pH 2·0. Because of this, ammonium sulphate fractionation at low pH proved to be the only feasible method in the final stages of purification.

As can be seen from Table 5, the powder obtained after stage III contained nearly 50 % of the original coagulase in a form which could be stored until needed. The subsequent low overall yield was due to the heavy loss in fraction V (i), where some 22 % of the initial coagulase contained a protein impurity which could not be eliminated by subsequent fractionation. Fraction V (ii), 5·6 % of the original coagulase, contained a single protein impurity which was easily removed by dialysis at pH 5·2.

The purified material obtained in the present study is the extracellular free coagulase which clots all fibrinogens, but only in the presence of a plasma activator. A certain specificity has been noted in this reaction, inasmuch as the free coagulase produced by a certain staphylococcal strain may react more readily with the plasma activator of one species than with another. Thus the free coagulase produced by strains of human origin clots bovine fibrinogen most readily in the presence of small amounts of human, rabbit and horse plasma, whereas clotting may not occur if sheep, guinea-pig, cow, dog or mouse plasma is used (Duthie, 1954*a*). In contrast with free coagulase, the majority of pathogenic staphylococci have on their surface a second factor which adsorbs the fibrinogen of certain species on to the cell surface, causing the cocci to adhere in gross floccules when shaken. This has been termed bound coagulase or clumping factor (Duthie, 1954*a*, 1955) and bears no relationship to free coagulase as far as can be discovered. It acts directly on the fibrinogens of certain species, and these are often not those whose plasma is clotted by the free coagulase produced by the same cells. It is antigenically distinct, and strains are easily found which produce one kind of coagulase and not the other. In the present studies variants of the Newman strain regularly appeared on subculture in 2 % (w/v) Bacto-tryptone medium. These were coated with bound coagulase, but produced no free coagulase either on fibrinogen plates or in the medium.

The conception that there are two distinct coagulases has been recently challenged by Jacherts (1956) on somewhat inadequate grounds. He made one preparation of extracellular protein and two of protein extracted from bacterial cells, all of which had coagulase activity, which he found were identical in biological, immunological and physical properties. From this he concluded that free and bound coagulase as defined by Duthie (1955) are also identical. This conclusion is, however, based on a misinterpretation of the results. Washed staphylococcal cells possess a small amount of cell-associated hyaluronidase (Rogers, 1954), which can be obtained by crushing the cells with Ballotini beads or merely by storage in buffer overnight at 0–4°, when release occurs. Small amounts of cell-associated free coagulase were obtained by him in the same way. In the present study 0·1 unit of free coagulase was obtained on crushing the washed cells of 100 ml. of a fully grown digest-broth culture and then extracting at pH 2·0. A very much larger amount (3·3 units) was obtained by extracting a similar volume of washed cells for a few minutes at pH 11·0 in the cold. Both extracts were identical with free coagulase and were quite distinct from bound coagulase. It is concluded that the material obtained by Jacherts was cell-associated free coagulase liberated by the treatment used, and that this bore no relationship to the bound coagulase described by Duthie (1955).

SUMMARY

1. The conditions under which free coagulase of high specific activity is liberated in the culture fluid have been investigated. Optimum conditions were obtained when a heavy inoculum of a fully grown digest-broth culture was seeded into a casein-hydrolysate medium and shaken for 1 hr. 20 min. at 37°.

2. The enzyme was isolated in an electrophoretically homogeneous form by adsorption on to cadmium sulphate, followed by fractional precipitation with ammonium sulphate.

3. On electrophoresis the purified enzyme had an isoelectric point of about pH 5·3. Ultracentrifuging a slightly impure preparation showed a minimum mean molecular weight of approximately 44 000.

4. The material was extremely toxic when injected intravenously into rabbits, and a concentration of 75 $\mu\mu$g./ml. clotted human plasma in 24 hr.

Our best thanks are due to Dr H. J. Rogers for many helpful discussions and for laboratory facilities, to Dr P. A. Charlwood for examination of material in the ultracentrifuge at the National Institute for Medical Research, Mill Hill, London, and to Glaxo Laboratories, Greenford, Middlesex for laboratory facilities. We also wish to thank Mr J. W. Ottewill and Mr W. Phillippe for their valuable help in constructing some of the equipment used. The work was made possible by a research grant from the South West Metropolitan Regional Hospital Board.

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The Phosphotransferase Activity of Phosphatases

1. SPECTROPHOTOMETRIC METHODS FOR THE ESTIMATION OF SOME PHOSPHATE ESTERS AND OTHER COMPOUNDS

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For the kinetic study of the 'phosphotransferase' (Dixon, 1949; Dixon & Webb, 1953) reaction catalysed by phosphatases (Morton, 1952, 1955*a*, 1958*a*, *b*) it became necessary to estimate phosphate esters without separation from the reaction mixture. It was required that the methods used would be accurate, sufficiently sensitive to measure between 0·05 and 0·2 μmole of a compound, and convenient enough to enable a large number of determinations to be handled.

Methods based on reactions catalysed by highly specific dehydrogenases appeared to meet all the above criteria; dehydrogenation of a substrate is coupled with the reduction of cytochrome *c* or diphosphopyridine nucleotide, which is estimated by changes in light-absorption at appropriate wavelengths. This paper describes the estimation of D-hexose phosphates, L-α-glycerophosphate, D-glyceraldehyde phosphate, dihydroxyacetone phosphate and phosphocreatine. Extension of these methods enables succinate, L-lactate and a variety of other compounds to be estimated.

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MATERIALS

Enzyme preparations

Yeast alcohol dehydrogenase. The crystalline enzyme was prepared from dried Fleischman's yeast by the method of Racker (1950).

Rabbit muscle L-α-*glycerophosphate dehydrogenase.* The preparation was based on the observations of Green (1936). Rabbit skeletal muscle (about 300 g.) was chilled in crushed ice immediately after removal from the animal, and finely minced and extracted twice (30 and 15 min.) with two

portions of about 300 ml. of 3 mM-KOH. The suspension was filtered through muslin and the filtrate used as source of the rabbit-muscle fractions A and B (see below).

The residual muscle was washed three times by suspending in about 1 l. of water at about 2°, and filtering through muslin and then squeezing the retained material.

The residue was ground with about 250 g. of acid-washed sand in a prechilled earthenware mortar with addition of about 100 ml. of 0·2 N-Na_2HPO_4. The paste was dispersed in 2 vol. of water at 0° and then centrifuged at 2500 *g* for 30 min. at 0–4°. The supernatant was cooled to 0°, then centrifuged at 14 000 *g* for 1 hr. at 0–4°. The precipitate was dispersed with a Potter–Elvehjem-type homogenizer in 10 ml. of 0·15 M-NaCl and the suspension was adjusted to pH 7·5 with 0·1 N-NaOH. The dispersion was again centrifuged at 14 000 *g* for 1 hr. at 0–4° and the precipitate redispersed in about 3 ml. of 0·15 M-NaCl at 0°. The suspension was stored at 0°. Activity rapidly declined after about 3 days.

Rabbit-muscle fractions A and B. The alkaline extracts from the rabbit skeletal muscle (see above) were combined. Fraction A was prepared from this extract as described by Racker (1947) for the 'glycerophosphate dehydrogenase and aldolase' fraction. Fraction B was also prepared from the same extract by Slater's (1953) modifications of Racker's (1947) 'phosphohexokinase' preparation. Fractions A and B were stored and used as described by Slater (1953).

Rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase. Crystalline enzyme was prepared from rabbit muscle essentially as described by Cori, Slein & Cori (1948). The crystals were collected by centrifuging at 10 000 *g* for 10 min. at 0–4° and dissolved in 0·03 M-sodium pyrophosphate–HCl buffer, pH 8·0, just before use.

Creatine phosphokinase. This was kindly supplied by Dr B. A. Askonas.

Reduced diphosphopyridine nucleotide–cytochrome c reductase. Initially, a suspension of microsomes from calf intestinal mucosa (Morton, 1954; Bailie & Morton, 1955) was used. The sedimented microsomes were suspended in 0·05 M-sodium phosphate buffer, pH 7·4, with a Potter–Elvehjem-type homogenizer, and centrifuged at 3000 *g* for 30 min. at 0°. The cloudy supernatant was stored at 0°.

In later work the enzyme was prepared from pig-heart muscle according to Mahler, Sarkar, Vernon & Alberty (1952), up to the first ammonium sulphate precipitation. The precipitate was dissolved in 0·05 M-glycylglycine buffer, pH 7·4, containing 0·5% of serum albumin, and stored at −14°.

Yeast lactic dehydrogenase. The crystalline preparation of Appleby & Morton (1954, and in preparation) was used. The crystals were washed with 0·05 M-sodium lactate, and dissolved in 0·5 M-NaCl and then dialysed anaerobically against a solution containing 0·5 M-NaCl, 0·05 M-sodium pyrophosphate–HCl buffer, pH 6·6, and 10 μM-sodium ethylenediaminetetra-acetate.

Muscle lactic dehydrogenase. This was prepared from rabbit skeletal muscle according to Askonas (1951).

Fructose diphosphatase. This was prepared from ox kidney as described by Morton (1958*a*).

Chemicals

Diphosphopyridine nucleotide (DPN), about 33% pure, and adenosine triphosphate (ATP) prepared by the late Mr J. Morgan were used in the initial studies. The ATP present in the DPN seriously interfered with some of the determinations (see later), and was converted into other compounds by hydrolysis in HCl (pH 1·5) for 4 min. at 100°. The solution was then cooled and adjusted to pH 6·8 with N-NaOH.

In later work DPN (92% pure) from the Sigma Chemical Co. was used.

Reduced diphosphopyridine nucleotide (DPNH) was prepared by enzymic reduction of DPN as described by Racker (1950).

Cytochrome c. This was prepared from horse heart or ox heart according to Keilin & Hartree (1945).

Phosphate esters. Previous papers (Morton, 1955*b*, *c*) give the sources of these compounds.

METHODS AND RESULTS

Measurement of phosphate transfer

Preliminary studies. Investigation of the kinetics of hydrolysis of phenyl phosphate (Morton, 1952, 1957) had shown that substrates of alkaline phosphatases are hydrolysed very slowly, or not at all, at about pH 9·5 (at 37°) and if the substrate is low in concentration (about 0·1 mM to 10 μM). It therefore appeared possible that conditions could be chosen such that any new ester synthesized by a phosphotransferase reaction (1) would be only slowly hydrolysed by the phosphatase (2), whereas hydrolysis of the initial substrate by the phosphatase (3) would proceed quite rapidly.

$$RO\cdot PO_3^{2-} + R'O\cdot H \rightarrow RO\cdot H + R'O\cdot PO_3^{2-} \quad (1)$$

$$R'O\cdot PO_3^{2-} + HO\cdot H \rightarrow R'O\cdot H + HO\cdot PO_3^{2-} \quad (2)$$

$$RO\cdot PO_3^{2-} + HO\cdot H \rightarrow RO\cdot H + HO\cdot PO_3^{2-} \quad (3)$$

The methods described by Axelrod (1948), Appleyard (1948), Meyerhof & Green (1950) and by Hanes & Isherwood (1949) were found unsuitable for estimation of small quantities of ester phosphate (e.g. glucose 6-phosphate) in the presence of large amounts of donor phosphate (e.g. phosphocreatine). However, it was found that with glucose, fructose or glycerol as the acceptors for the transferred phosphate group the product formed is capable of estimation by specific and sensitive enzymic methods.

General procedure. Glass tubes (approx. 6 mm. × 60 mm.) were marked at 0·35, 0·4 and 0·45 ml. and at 2 ml. volumes. Rubber stoppers for the tubes were extracted with warm 5% (w/v) trichloroacetic acid and then with boiling water. The stoppers were immersed in boiling water and dried immediately before use.

The acceptor was weighed into a tube and buffer, phosphate donor and magnesium salt were added. The acceptor was dissolved and the volume was adjusted to 0·35, 0·4 or 0·45 ml. (as appropriate) with water. The tube was brought to 38° in a water bath and enzyme (0·05–0·1 ml.) was added. The stopper was immediately inserted, the contents of the tube were thoroughly mixed by inversion and the tube was replaced in the water bath. This method was

used since some of the solutions, such as 4M-glucose and 5M-glycerol, were very viscous. After the reaction period (usually 5 min. at 38°), 0·1 ml. of 20% (w/v) trichloroacetic acid was added, the stopper was re-inserted and the contents of the tube were mixed for 15 sec. and then adjusted to pH 8·0 with a predetermined volume of 0·5N-NaOH. The volume was then adjusted to 2 ml. with water, and the tube stoppered and the contents were mixed. The tube was then held in an ice bath. Suitable portions (0·1–0·5 ml.) were used for estimation of inorganic phosphate, phenol or creatine (according to the donor used) and acceptor phosphate formed. Enzyme was added to the control tube immediately after the trichloroacetic acid.

Unless otherwise specified, all estimations of newly formed phosphate esters were carried out with a Beckman Model DU spectrophotometer at room temperature with quartz cuvettes (1 cm. light path).

Estimation of phosphorylated compounds

Fructose 1:6-*diphosphate*, D-*glyceraldehyde* 3-*phosphate and dihydroxyacetone phosphate.* Procedure A of Slater (1953) was followed. There was no reaction with added glucose 1-phosphate, fructose 6-phosphate or fructose 1-phosphate. Some preparations reacted with glucose 6-phosphate owing to contaminant phosphohexokinase. This was removed by dissolving the enzyme paste in a minimum of water at 0°, and adjusting the pH to about 6·0 and holding for 30 min. After readjusting to pH 7·5, the mixture was filtered through a thin layer of Hyflo Super-Cel and the filtrate was stored at 0° until used.

Hexose monophosphates. Procedure B (a) of Slater (1953) was used. This procedure estimates glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate, as well as hexose diphosphate which, however, may be estimated separately as above. Synthetic fructose 1-phosphate reacted rather slowly in this system. However, an estimate of fructose 1-phosphate was obtained by using about three times the normal amount of rabbit-fractions A and B. Because of the relatively high blank value, however, this estimate is less accurate than that for the other compounds mentioned.

With very high concentrations of glucose, there was a continuous slow oxidation of reduced DPN. However, extrapolation to obtain the true change in optical density (see Slater, 1953, fig. 1) enabled an accurate determination of the hexose monophosphate present to be made.

Differentiation of fructose diphosphate, glyceraldehyde phosphate and dihydroxyacetone phosphate. Slater's (1953) procedure A estimates the sum of fructose diphosphate and triose phosphates. It was found that the amount of fructose diphosphate in a mixture containing triose phosphates could be estimated from the loss in reactive component after hydrolysis catalysed by the specific fructose diphosphatase of ox kidney (Gomori, 1943). Slater's procedure A was used for the determinations since the products of hydrolysis of fructose diphosphate by the kidney enzyme are fructose 6-phosphate and inorganic phosphate.

The D-glyceraldehyde phosphate in the same mixture was then specifically estimated by measuring the reduction of DPN at 340 mμ in the presence of sodium arsenate and glyceraldehyde phosphate dehydrogenase essentially as described by Cori *et al.* (1948).

Phosphocreatine. This was estimated with creatine phosphokinase and procedure B (b) as described by Slater (1953).

L-α-*Glycerophosphate.* The method depends on the reduction of heart-muscle ferricytochrome *c* by the particle-bound L-α-glycerophosphate dehydrogenase of skeletal muscle according to reaction 4:

L-α-Glycerophosphate + ferricytochrome *c* →
dihydroxyacetone phosphate + ferrocytochrome *c* (4)

This reaction is catalysed by an enzyme which is firmly attached to lipoprotein particles and which, at the time of these studies, had not been obtained in true solution.

Four cuvettes are used for the estimation. The first contains 1·5 μmoles of KCN (at pH 8·0), 0·1 μmole of ferricytochrome *c*, 30 μmoles of $MgCl_2$, 150 μmoles of glycylglycine buffer, pH 8·0, about 0·2 ml. of enzyme and 0·2–0·5 ml. of the test solution to bring the volume to 3 ml. The test solution is omitted from the second cuvette, cytochrome from the third and enzyme from the fourth. Water replaces the missing component. Optical densities were measured before and after addition of the final components. The contents of the cuvettes were mixed with plastic stirrers (see Dixon, 1954) before all readings, which were continued until there was no change between successive 10 min. readings.

Fig. 1 shows a typical determination. E_2 and E_3 are the initial optical densities (corrected for the dilution on adjustment to 3 ml. volume) and E'_2 and E'_3 the extrapolated

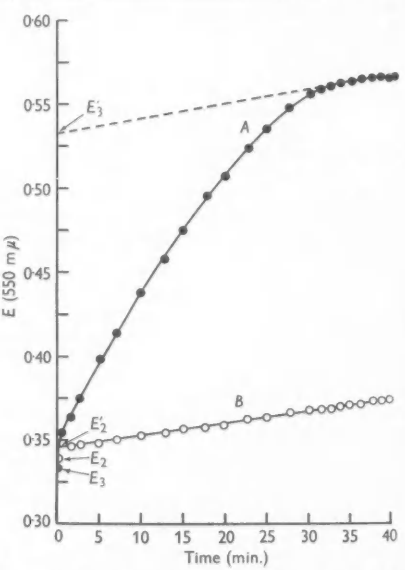

Fig. 1. Estimation of α-glycerophosphate with rabbit-muscle cytochrome *c*-linked L-α-glycerophosphate dehydrogenase and cytochrome *c*. E_2 and E_3 are the initial optical densities (corrected for the dilution on adjustment to 3 ml. volume) and E'_2 and E'_3 the extrapolated densities for the control (curve *B*) and test (curve *A*) respectively. Other details are given in the text.

optical densities for the control and test cuvettes respectively at the completion of the reaction (cf. fig. 1, Slater, 1953). The change in optical density (ΔE_{550}) due to the compound being estimated thus equals $(E'_3 - E_3)$–$(E'_2 - E_2)$ When the final volume of reactants is 3 ml., the amount (in μmoles) of L-α-glycerophosphate is

$$0{\cdot}5 \times (E_{550} \times 0{\cdot}154),$$

since 2 moles of cytochrome *c* are reduced for each mole of L-α-glycerophosphate. The factor 0·154 was calculated from the molar-extinction coefficients of reduced and oxidized cytochrome *c* (see Theorell, 1936).

The recovery of added DL-α-glycerophosphate from 0·05 to 0·2 μmole/cuvette was between 90 and 94%.

Although the enzyme preparation is substantially free of ferrocytochrome *c* oxidase, normally KCN (0·5 mM) was included in the reaction mixture as a precaution against oxidation of the cytochrome. Potassium ferricyanide and 2:6-dichlorophenol indophenol as hydrogen acceptors were found to be unsatisfactory for the microdetermination of α-glycerophosphate. Neither glycerol (up to 4 M) nor β-glycerophosphate caused inhibition of the enzyme and did not interfere with the estimations of α-glycerophosphate.

However, inorganic phosphate at concentrations above 0·02 M caused substantial inhibition of the reduction of cytochrome *c* (Morton, 1952).

L-α-Glycerophosphate was later estimated by the coupling of the DPN-linked muscle L-α-glycerophosphate dehydrogenase (reaction 5) with the specific reduced DPN–cytochrome *c* reductase (reaction 6) thus:

L-α-Glycerophosphate + DPN^+
$\rightleftharpoons$ dihydroxyacetone phosphate + DPNH + H^+ (5)

DPNH + H^+ + 2 ferricytochrome *c*
$\rightarrow$ DPN^+ + 2 ferrocytochrome *c* (6)

The overall reaction will proceed to complete reduction of L-α-glycerophosphate since the equilibrium of the reductase reaction is displaced very much in favour of oxidation of reduced DPN.

Four cuvettes (0·5 cm. light path) were used for the estimation. The first contained (in 1·2 ml.) 60 μmoles of glycylglycine buffer, pH 8·5; 0·08 μmole of ferricytochrome *c*; 0·2 μmole of DPN; 0·05 ml. of rabbit-muscle fraction A (0·5 ml. of paste diluted to 3 ml. with water); 0·025 ml. (approx. 100 μg. of enzyme protein) of reduced DPN–cytochrome *c* reductase from pig-heart muscle and the test solution. The second cuvette omitted the test solution, the third the cytochrome, and from the fourth the enzymes were omitted. Water replaced the missing components. The reaction was started by the addition of the two enzymes. The optical density (at 550 mμ) was determined before and after additions of the enzymes, and measurements were continued (usually for 25 min.) until there was no change between successive 10 min. readings, or the rate of change in the cuvette containing the test solution was the same as that in the control. The amount of L-α-glycerophosphate was calculated from the change in optical density due specifically to the test solution. Fig. 2 shows a typical determination. As previously, ΔE_{550} equals $(E'_3 - E_3)$–$(E'_2 - E_2)$. Recoveries of added DL-α-glycerophosphate (0·08–0·25 μmole/cuvette) of 86–92% were obtained.

In some studies, 0·1 ml. of a suspension of intestinal microsomes (about 4 mg. dry wt.) was used instead of purified heart-muscle cytochrome *c* reductase, 60 μmoles of sodium phosphate buffer, pH 7·8, replaced the glycylglycine and the reaction mixture contained 1 μmole of KCN. This system was less satisfactory.

Estimation of other compounds

Although not used in the investigation of the phosphotransferase reaction, some procedures are described here which have been found useful for the spectrophotometric estimation of succinate and lactate in pure solution.

Succinate. The preparation of cytochrome *c*-linked α-glycerophosphate dehydrogenase from skeletal muscle also contains an active succinic dehydrogenase system which catalyses reaction 7. Succinate was estimated by a similar method to that used for estimation of α-glycerophosphate. The recovery of 0·03 μmole of sodium succinate/cuvette was 94%.

Succinate + ferricytochrome *c*
$\rightarrow$ fumarate + ferrocytochrome *c* (7)

Lactate. L-Lactate was estimated by measurement of the reduction of ferricytochrome *c* (reaction 8) catalysed by yeast lactic dehydrogenase (cytochrome b_2). The method was similar to that used for estimation of α-glycerophosphate except that the yeast lactic dehydrogenase replaced the muscle glycerophosphate dehydrogenase, and sodium pyrophosphate–HCl buffer, pH 8·0, replaced the glycylglycine buffer.

L-Lactate + ferricytochrome *c*
$\rightarrow$ pyruvate + ferrocytochrome *c* (8)

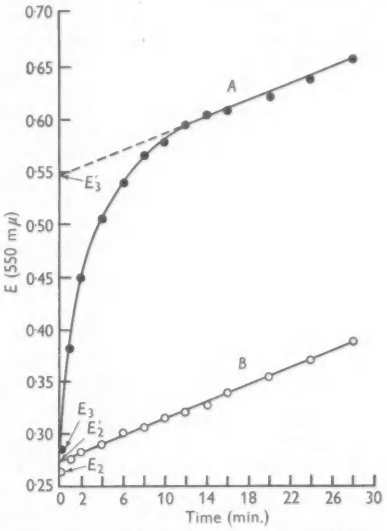

Fig. 2. Estimation of α-glycerophosphate with rabbit-muscle DPN-linked L-α-glycerophosphate dehydrogenase, reduced DPN–cytochrome *c* reductase and cytochrome *c*. E_2 and E_3, and E'_2 and E'_3 have the same meaning as in Fig. 1 for the control (curve *B*) and test (curve *A*) respectively. Other details are given in the text.

DISCUSSION

For estimation of products of a phosphotransferase reaction, it seemed desirable to avoid, if possible, separation of the newly formed phosphate ester from the other reactants. Specific, sensitive enzymic methods for estimation of phosphate esters were therefore used for kinetic studies of phosphate transfers catalysed by acid and alkaline phosphatases (Morton, 1958*a*, *b*).

The methods developed by Slater (1953) are particularly valuable in that a number of different compounds can be estimated with the same enzyme preparations. Some of the methods described here are extensions of Slater's procedures. The differentiation of fructose diphosphate from the triose phosphates, and the separate estimation of glyceraldehyde phosphate, for example, should add to the usefulness of Slater's procedures. These methods have not, as yet, been used in the analysis of tissue extracts. However, it would be expected that they would be quite satisfactory for this purpose. Unfortunately, the product of hydrolysis of fructose 1:6-diphosphate by fructose diphosphatase is fructose 6-phosphate, which will react in Slater's procedure B(a). Hence fructose diphosphatase cannot be used for differentiation of hexose monophosphates and fructose diphosphate. Where both of these are present in a mixture, the amount of hexose monophosphate must be calculated from the difference between separate determinations by Slater's procedures A and B(a). Glucose 6-phosphate, of course, is readily estimated with triphosphopyridine nucleotide and yeast glucose 6-phosphate dehydrogenase as described by Kornberg & Pricer (1951).

The sensitivity of the spectrophotometric methods described here is conveniently increased by almost threefold by reducing the volume of the cuvette (1 cm. light path) to 1·2 ml. with the insert described by Martin & Morton (1956).

Estimations based on enzymic reduction of cytochrome c

Estimation of enzyme substrates with cytochrome *c* as the hydrogen (or electron) acceptor are particularly sensitive because of the high molar extinction coefficient of ferrocytochrome *c* (28×10^3 at 550 mμ; Theorell, 1936; Keilin & Slater, 1953). Moreover, two molecules of cytochrome *c* are reduced for each molecule of substrate oxidized. The reactions proceed to completion since the equilibria of the cytochrome *c*-linked systems are very greatly in favour of reduction of cytochrome *c*. Hence the choice of the cytochrome *c*-linked α-glycerophosphate dehydrogenase for estimation of α-glycerophosphate (Morton, 1952, 1955*a*). Estimation of this compound by determining the amount of DPN reduced with the DPN-linked α-glycerophosphate dehydrogenase of muscle in the presence of a trapping agent for dihydroxyacetone phosphate was found to be unsatisfactory (Morton, 1952). Bublitz & Kennedy (1954), however, recently reported successful estimations with this enzyme.

In addition to α-glycerophosphate, succinate and lactate, with the appropriate cytochrome *c*-linked dehydrogenases, choline, xanthine and hypoxanthine, probably could be estimated by similar procedures to those described here. However, the range of substrates which could be estimated spectrophometrically is greatly extended by the coupling of the reactions of the DPN-linked dehydrogenases with the reaction catalysed by reduced DPN–cytochrome *c* reductase. L-α-Glycerophosphate and L-lactate have both been successfully estimated. However, the method is not as satisfactory as was at first anticipated. With the reduced DPN–cytochrome *c* reductase of microsomes, difficulties arise due to changes in turbidity during the course of the reaction. Although the enzyme extracted from heart muscle is readily soluble, trouble has been found owing to a slow non-specific reduction of cytochrome *c* catalysed by this preparation. Moreover, the enzyme is rather unstable at room temperature, and is inhibited by a variety of metal salts (see Vernon, Mahler & Sarkar, 1952). Conditions for estimation of any particular substrate require extensive investigation.

Glock & McLean (1955) have used the coupled reaction of alcohol dehydrogenase and reduced DPN–cytochrome *c* reductase of heart muscle for estimation of DPN in tissue extracts. These workers measure the rate of reduction of cytochrome *c* under carefully standardized conditions, rather than the total amount of cytochrome *c* reduced, possibly because of difficulties due to non-specific reduction of cytochrome *c*.

SUMMARY

1. Methods are described for kinetic study of the phosphotransferase reaction. The phosphate esters synthesized by phosphate transfer are estimated spectrophotometrically with substrate-specific enzymes. The methods avoid the separation of the phosphate esters from the other components of the reaction mixtures.

2. Slater's (1953) procedures were used for estimation of triose phosphates and hexose phosphates. Extensions of these procedures for differentiation of fructose diphosphate, glyceraldehyde phosphate and dihydroxyacetone phosphate are described.

3. Methods for estimation of L-α-glycerophosphate, succinate and L-lactate are described.

Cytochrome *c* is used as the hydrogen acceptor with the appropriate cytochrome *c*-linked dehydrogenase system, or with the appropriate diphosphopyridine nucleotide-linked dehydrogenase together with reduced diphosphopyridine nucleotide-cytochrome *c* reductase.

I wish to thank Professor E. C. Slater for suggesting the use of enzymic methods for estimation of the products of phosphate transfer to hexoses, and for making available in 1951 the details of his procedures before publication. I am most grateful to Dr M. Dixon, F.R.S., for his valuable guidance and interest in this work, which was carried out in 1951 with the financial assistance of the Agricultural Research Council of Great Britain. The assistance of the Council is gratefully acknowledged.

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The Phosphotransferase Activity of Phosphatases

2. STUDIES WITH PURIFIED ALKALINE PHOSPHOMONOESTERASES AND SOME SUBSTRATE-SPECIFIC PHOSPHATASES

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The synthesis of organic phosphates by enzymic transfer of the phosphate group from organic 'donor' compounds (such as *p*-nitrophenyl phosphate) to a suitable alcohol (such as propan-1-ol) was first demonstrated by Axelrod (1947, 1948*a*) and by Appleyard (1948). These workers used crude acid phosphatase preparations from citrus fruits and from prostate gland respectively. In both cases there was net synthesis of new ester. Much earlier Kay (1928) had shown that alkaline phosphatases may catalyse synthesis of an organic ester from an alcohol and inorganic phosphate, but Axelrod (1948*b*) established that the biosynthesis catalysed by the citrus-fruit acid phosphatase preparation did not involve the intermediary formation of inorganic phosphate. Meyerhof & Green (1950) subsequently showed that partially purified alkaline phosphatase from calf intestinal mucosa catalysed an exchange reaction between the phosphate group of phosphocreatine and that of glycerophosphate.

These workers all showed that hydrolysis of the donor phosphate accompanied synthesis of the new ester. However, it was by no means certain that the same enzyme catalysed both reactions. Appleyard (1948) had proposed that a 'transphosphorylase', distinct from the phosphatase, was present in the extract of prostate gland. In 1949 therefore investigations were initiated to establish

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the nature of the enzyme catalysing this 'phosphotransferase' (Dixon, 1949) reaction.

As an essential step in this investigation, alkaline phosphatases were purified from cow's milk (Morton, 1953*a*) and from calf intestinal mucosa (Morton, 1954).

The properties of the purified enzymes were described (Morton, 1955*a*), including the substrate specificity (Morton, 1955*b*) and the kinetics of hydrolysis of a typical substrate (Morton, 1957). Sensitive spectrophotometric methods were then developed to enable the accurate estimation of selected products of transferase activity without separation from the reaction mixture (Morton, 1952*a*, 1958*a*). The results of the investigation described here show that the phosphotransferase activity is a property of both purified alkaline phosphatases, and provide some information on the mechanism of the reaction.

In contrast with the findings with the purified alkaline phosphatases, no phosphotransferase activity was detected in partially purified preparations of hexose diphosphatase of ox kidney, 5′-nucleotidase of bull testes or myosin–adenosine triphosphatase of rabbit skeletal muscle.

Certain aspects of this work have been published (Morton, 1952*b*, *c*; 1953*b*, 1955*c*).

MATERIALS AND METHODS

Organic phosphates. The sources of these are given in previous papers (Morton, 1955*a*, *b*).

Other chemicals. Redistilled glycerol was kindly supplied by Dr G. Weber. DL-Glyceraldehyde was a gift from Dr D. M. Needham, F.R.S.

Alkaline phosphatases. These were purified from cow's milk (Morton, 1953*a*) and from calf intestinal mucosa (Morton, 1954). Solutions of enzymes were prepared from freeze-dried powder which had been stored over $CaCl_2$ at 0°. The specific activities had declined somewhat from those obtained immediately after purification. The activities with β-glycerophosphate were not regularly tested, but may be calculated approximately from the relationship that 1 μg. of intestinal phosphatase is equivalent to 7 units, and 1 μg. of milk phosphatase to 1·5 units, of activity as previously defined (Morton, 1953*c*).

Alkaline hexose diphosphatase. Minced ox kidney (100 g.) was extracted with sodium lactate buffer (pH 4·2 at 18°) as described by Gomori (1943). The insoluble protein was removed by centrifuging at about 1800 *g* for 30 min. The supernatant was adjusted to pH 7·5 with 0·1 N-NaOH and then filtered through a thin layer of Hyflo Super-Cel over no. 1 Whatman paper on a Büchner funnel.

The clear filtrate was fractionated with $(NH_4)_2SO_4$. The fraction obtained between 55 and 68% saturation was dissolved in 5 ml. of water, and dialysed in seamless cellulose tubing against several changes of glass-distilled water at 0° for 48 hr. and then centrifuged at 14 000 *g* for 30 min. at 0–2°. The supernatant containing the enzyme was stored at 0°. Before use, the enzyme was activated by preincubation in 0·01 M-veronal (sodium diethylbarbiturate)–HCl buffer, pH 9·2, containing 0·01 M-cysteine hydrochloride and 0·01 M-$MgCl_2$. At 38° approx. 40 μg. of inorganic phosphate was liberated/min./mg. dry wt. of enzyme from 5 mM-fructose 1:6-diphosphate in 0·05 M-veronal–Na_2CO_3, pH 9·5, containing mM-cysteine hydrochloride and mM-$MgCl_2$. There was no hydrolysis of 0·01 M-β-glycerophosphate under similar conditions.

5′-Nucleotidase. The mince from two decapsulated bull's testes was placed in a muslin bag and washed in running tap water until the effluent was colourless. The mince was then extracted with 0·15 M-NaCl (pH 7·5 at 20°) for 20 min. The extract was centrifuged at 2500 *g* for 30 min. and the resulting supernatant was centrifuged at 14 000 *g* for 1 hr. The active protein was precipitated from the supernatant by addition of solid $(NH_4)_2SO_4$ to 50% saturation, and collected by centrifuging, then dissolved in water and dialysed in seamless cellulose tubing against 0·15 M-NaCl. The material was then centrifuged at 14 000 *g* for 20 min. The active supernatant was colourless but opalescent. It was stored at 0°. Whereas 0·1 ml. of the enzyme solution liberated about 3 μg. of inorganic phosphate/min. from 5 mM adenosine-5′-phosphate (AMP) in 0·02 M-veronal–HCl buffer, pH 9·0, containing 0·01 M-$MgCl_2$, there was no inorganic phosphate liberated from 0·01 M-β-glycerophosphate under similar conditions.

Myosin–adenosine triphosphatase. Myosin, prepared from rabbit muscle as described by Tsao (1953), was kindly supplied by Dr T. C. Tsao.

Other enzymes. The preparation of the other enzymes used is described in the preceding paper (Morton, 1958*a*).

Inorganic phosphate. This was estimated according to Weil-Malherbe & Green (1951).

Phenol. This was estimated by the procedure of King (1951) modified as previously described (Morton, 1955*b*).

Creatine. This was estimated by the method of Eggleton, Elsden & Gough (1943).

pH *values.* These were estimated in replicate tubes of those used for the enzyme reactions. Unless otherwise specified, all values given refer to pH at 38°.

Estimation of phosphate transfer. The procedure is described in the preceding paper (Morton, 1958*a*).

RESULTS

Effect of phosphocreatine on the synthesis of glucose phosphate from glucose and inorganic phosphate

The progressive synthesis of glucose phosphates from sodium phosphate (0·16 M) and glucose (0·80 M) with intestinal and milk phosphatases was compared with that obtained with a similar reaction mixture but with the addition of phosphocreatine (14 mM). The results in Table 1 show that there was an increased synthesis with both enzymes in the presence of added phosphocreatine, but the additional glucose phosphate formed with phosphocreatine diminished with time. After prolonged incubation, the amount of hexose phosphate obtained with and without phosphocreatine was essentially the same.

In a further experiment, the amount of purified intestinal phosphatase was reduced to about one-

Table 1. *Synthesis of glucose phosphates by alkaline phosphatases from glucose and sodium phosphate, with and without phosphocreatine*

The reaction mixture (0·4 ml.) contained 0·80 M-glucose, 0·16 M-sodium phosphate, pH 8·5, approx. 90 μg. of intestinal phosphatase or about 200 μg. of milk phosphatase and, where added, 14 mM-phosphocreatine. The reaction was carried out at 38° and at pH 8·5.

Phosphatase	Time (hr.)	Glucose phosphate formed/tube (μmole): Without creatine phosphate	Glucose phosphate formed/tube (μmole): With creatine phosphate	Increased synthesis with creatine phosphate: (μmole)	Increased synthesis with creatine phosphate: (%)
Calf intestine	0	0	0	0	—
	0·1	0·007	0·027	0·02	290
	1	0·16	0·27	0·11	69
	2·5	0·32	0·41	0·09	28
	6	0·42	0·49	0·07	17
	25	0·96	—	—	—
Cow's milk	0	0	0	—	—
	0·1	0·003	0·023	0·02	660
	1	0·07	0·13	0·06	86
	2·5	0·27	—	—	—
	6	0·31	0·37	0·06	19
	25	0·61	0·63	0·02	3

Table 2. *Synthesis of glucose phosphates by alkaline phosphatase from glucose and phosphocreatine only*

The reaction mixture (0·12 ml.) contained 1·7 M-glucose, 0·03 M-veronal (sodium diethylbarbiturate)–HCl buffer, intestinal phosphatase (approx. 9 μg.) and either 0·1 M-sodium phosphate or 64 mM-phosphocreatine as indicated. The pH was adjusted to that shown. The reaction period was 5 min. at 38°.

pH	Glucose phosphate formed/tube (μmole): With inorganic phosphate	Glucose phosphate formed/tube (μmole): With creatine phosphate
7·5	0	0·072
8·5	0	0·074
9·0	0	0·084
9·3	0	0·087

tenth of that used in the former experiment, and the amount of glucose phosphate formed from glucose (1·7 M) and phosphocreatine (64 mM) was compared with that obtained from glucose (1·7 M) and sodium phosphate (0·1 M) at several pH values. Table 2 shows that, under the conditions used, there was no appreciable synthesis of glucose phosphate from glucose and inorganic phosphate, whereas there was considerable synthesis from glucose and phosphocreatine. There was no synthesis of glucose phosphate when the sodium phosphate concentration was 0·05 or 0·01 M. The reduced amount of intestinal phosphatase explains the failure to detect glucose phosphates formed from inorganic phosphate and glucose as shown in Table 2, whereas synthesis was found with a greater amount of enzyme (Table 1).

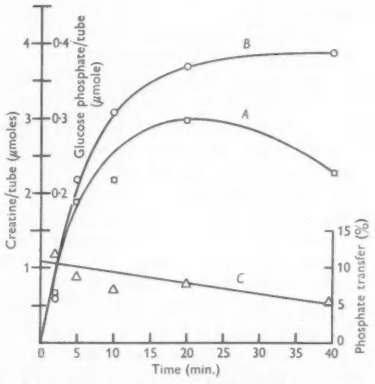

Fig. 1. Progress curve for synthesis of glucose phosphate by intestinal phosphatase at 38°. The reaction mixture (0·4 ml.) contained 1·5 M-glucose, 13 mM-phosphocreatine, 0·05 M-sodium bicarbonate–sodium carbonate buffer, pH 9·65, and about 18 μg. of intestinal phosphatase. *A*, Glucose phosphate; *B*, creatine; *C*, percentage transfer.

Kinetic studies of the transferase reaction

Estimation of initial velocity of phosphate transfer. Fig. 1 (curve *A*) shows that the rate of formation of glucose phosphate from phosphocreatine (13 mM) and glucose (1·5 M) at pH 9·65 is linear for about 4 min., after which it rapidly declines. After 20 min., when some 75 % of the phosphocreatine has been used, the glucose phosphate reaches a maximum value, and the amount present diminishes with further incubation, probably due to hydrolysis by the phosphatase. From Fig. 1 it appears that values close to the true initial rates of

glucose phosphate formation will be obtained if the amount of enzyme is adjusted so that the concentration of newly formed ester does not exceed 0·4 mM, namely, is less than 0·2 μmole/0·5 ml. of reaction mixture. These conditions were used in subsequent studies.

The percentage transfer is a measure of the efficiency of the phosphotransferase reaction. It was defined by Axelrod (1948*a*) as the quotient:

$$\frac{\text{moles of ester phosphate formed} \times 100}{\text{moles of donor phosphate utilized}}.$$

Fig. 1 (curve *C*) shows that the percentage transfer to glucose (1·5 M) is initially about 12%, but the value steadily diminishes during the course of the reaction. This clearly indicates that the accumulation of glucose phosphate is opposed by the hydrolysis catalysed by the phosphatase itself. The rate of hydrolysis of the glucose phosphate formed will increase with increasing concentration of this compound, as has been shown for other ester phosphates (Morton, 1952*a*, 1957) and is apparent from Fig. 1. To be valid therefore estimates of percentage transfers should be based on measurements of initial rates.

Effect of enzyme concentration. The rates of hydrolysis of phosphocreatine and of synthesis of glucose phosphate were found to be proportional to the amount of enzyme, provided that the conditions were such that the initial rates were reliably estimated. The percentage transfer was then independent of the amount of enzyme.

Influence of pH *on the transferase reaction.* The reaction was studied over the range pH 7·9–10·1. The results for intestinal phosphatase (Fig. 2, curve *A*) show that, in the absence of glucose, the inorganic phosphate and the creatine liberated closely coincide. Addition of glucose (1·5 M) depresses the rate of hydrolysis of phosphocreatine, as indicated by the decreased amount of inorganic phosphate liberated (curve *C*). The excess of creatine above the inorganic phosphate liberated (cf. curves *B* and *C*) is due to the transferase reaction. The amount of glucose phosphate formed (curve *D*) so varies with the pH of the reaction that the percentage transfer (curve *E*) remains fairly constant over the pH range investigated.

The results obtained with milk phosphatase (Table 3) substantiate the findings obtained with intestinal phosphatase.

Effect of the concentration of phosphate donor. In different experiments, the concentration of phosphocreatine has been varied from 5 to 60 mM at similar glucose concentrations (1·5 M). The initial velocity of glucose phosphate formation increased at the higher donor concentrations, but the percentage transfer was not changed significantly over this range. It appears therefore that the hydrolytic and transferase reactions are similarly influenced by the concentration of the phosphate donor.

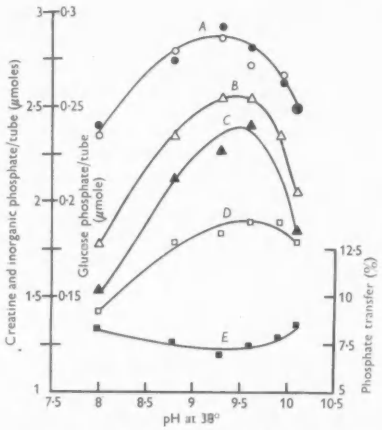

Fig. 2. Influence of pH on synthesis of glucose phosphate. The reaction mixture (0·4 ml.) contained 13 mM-phosphocreatine, 0·01 M-magnesium acetate, 0·1 M-veronal-sodium acetate–HCl or 0·05 M-sodium bicarbonate-sodium carbonate buffer, about 9 μg. of intestinal phosphatase and, where added, 1·5 M-glucose. The reaction period was 5 min. at 38°. *A*, Creatine (○) and inorganic phosphate (●) in the absence of glucose; other curves with 1·5 M-glucose; *B*, creatine; *C*, inorganic phosphate; *D*, glucose phosphate; *E*, percentage transfer.

Table 3. *Influence of* pH *on the synthesis of glucose phosphate by phosphate transfer catalysed by milk alkaline phosphatase*

The reaction mixture (0·4 ml.) contained 0·03 M-sodium bicarbonate–sodium carbonate buffer at the pH shown; 0·01 M-magnesium acetate; 13 mM-phosphocreatine; 1·5 M-glucose; 20 μg. of milk phosphatase. The reaction was for 5 min. at 38°. Figures in parentheses show the amounts of creatine and inorganic phosphate liberated in identical reaction mixtures without glucose.

	Product/tube (μmole)			
pH	Creatine	Inorganic phosphate	Glucose phosphate	Phosphate transfer (%)
8·75	1·27 (1·75)	1·04 (1·75)	0·10	7·8
9·55	1·38 (1·71)	1·18 (1·72)	0·11	8·0
10·05	1·13 (1·35)	1·00 (1·35)	0·08	7·1

Effect of acceptor concentration. The influence of increasing glucose concentrations on the phosphate-transfer reaction with phosphocreatine as donor is shown in Fig. 3. The rate of hydrolysis, as indicated by curve *B*, declines continuously as the concentration of the acceptor increases up to about

1·5M-glucose. Curve *A* shows that the rate of creatine liberation (due to both hydrolysis and transfer reactions) declines more slowly up to 1·5–2·0M-glucose. At higher glucose concentrations, however, both the liberation of creatine and of inorganic phosphate fall rapidly and in a similar fashion. The rate of glucose phosphate formation (curve *C*) increases with glucose concentration, reaches a maximum value near 1·5–2·0M-glucose, and then declines at higher glucose concentrations. The similar decline in the rates of creatine, inorganic phosphate and glucose phosphate formation above 2M-glucose indicates that the phosphatase is inhibited. The percentage transfer (curve *D*) increases with increasing glucose concentration and reaches a maximum value between 1·5 and 2·0M-glucose, which is unchanged by further increase of glucose concentration.

Acceptor specificity of intestinal alkaline phosphatase

A number of potential phosphate acceptors were examined.

Glucose. The glucose phosphate formed as shown in Fig. 3 is the sum of glucose 1- and glucose 6-phosphate, since Slater's (1953) procedure B(a) estimates both. Inorganic phosphate was removed from the reaction products formed with 2M-glucose (see Fig. 3) by addition of ammoniacal magnesia, and the esters were then precipitated as the barium salts. After washing and conversion into the ammonium salts, the products were applied to washed Whatman no. 1 paper and run in the propan-1-ol–aq. NH_3 soln. solvent of Hanes & Isherwood (1949). Glucose 6-phosphate was the only new ester detected with the procedure of Hanes & Isherwood (1949).

Fructose. Fig. 4 shows that fructose is also phosphorylated. Here, Slater's (1953) procedure B(a) estimates the sum of fructose 1:6-diphosphate and of fructose 6-phosphate. In one experiment a large amount of products was formed under the conditions of Fig. 4 but with a tenfold increase of enzyme. Fructose diphosphate was estimated separately from fructose 6-phosphate (see Morton, 1958*a*) and found to be between 2 and 5% of the total phosphates formed at 2 and 4M-fructose. With very large amounts of muscle fractions A and B, and with Slater's (1953) procedure B(a), the estimate of fructose phosphate differed very little from that obtained with the usual amount of muscle fractions, indicating that there is very little fructose 1-phosphate formed (see Morton, 1958*a*).

Glycerol. Fig. 5 shows that glycerol is readily phosphorylated. As both D- and L-isomers are formed, the glycerophosphate formed was estimated as twice the L-α-glycerophosphate determined enzymically as already described (Morton, 1958*a*). As shown in Fig. 5, there was substantial agreement between the amount of glycerophosphate estimated enzymically with that estimated from the difference between the liberated creatine and inorganic phosphate, indicating that very little β-glycerophosphate was formed in the reaction.

Fig. 5 shows the effect of increasing glycerol concentrations on the phosphotransferase reaction. The percentage transfer (curve *B*) reaches a maximum near 4M-glycerol and is unchanged by further increase of glycerol concentration.

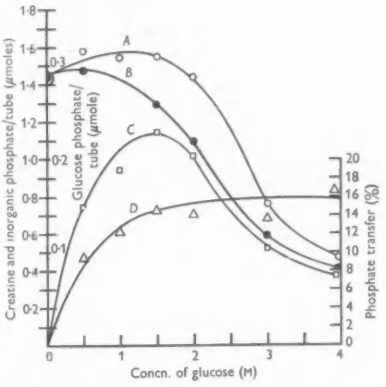

Fig. 3. Effect of glucose concentration on the phosphotransferase reaction. The reaction mixture (0·5 ml.) contained 26 mM-phosphocreatine; 0·01M-magnesium acetate; 0·05M-veronal–sodium carbonate buffer, pH 9·8; about 9 μg. of intestinal alkaline phosphatase; glucose at the concentrations shown. The reaction period was 5 min. at 38°. *A*, Creatine; *B*, inorganic phosphate; *C*, glucose phosphate; *D*, percentage transfer.

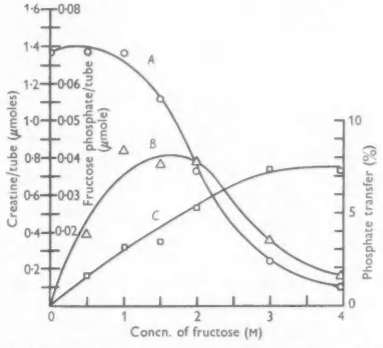

Fig. 4. Effect of fructose concentration on the phosphotransferase reaction. Conditions were as for Fig. 3 except that fructose replaced glucose. *A*, Creatine; *B*, fructose phosphate; *C*, percentage transfer.

Fig. 6 (curve *A*) shows the coupled reaction between intestinal alkaline phosphatase and cytochrome *c*-linked L-α-glycerophosphate dehydrogenase. Reduction of cytochrome *c* due to the formation of L-α-glycerophosphate was initiated by addition of intestinal phosphatase.

Propane-1:2-*diol*. Table 4 shows that propane-1:2-diol is a suitable acceptor. The product was estimated as the difference between creatine and inorganic phosphate liberated enzymically.

Glyceraldehyde and dihydroxyacetone. The phosphorylated derivatives of both trioses were estimated enzymically by Slater's (1953) procedure (Morton, 1958*a*). DL-Glyceraldehyde and dihydroxyacetone (both from L. Light and Co. Ltd.) were phosphorylated (Table 5). The product with glyceraldehyde was estimated as twice the triose phosphate determined enzymically, since L-glyceraldehyde phosphate would not be determined and both optical isomers would be expected.

Fig. 6 (curve *B*) shows the coupled reaction between alkaline phosphatase and diphosphopyridine nucleotide (DPN)-linked L-α-glycerophosphate dehydrogenase. Oxidation of reduced DPN was initiated by addition of phosphatase.

Sucrose. No detectable amount of new ester was formed with sucrose whereas hexose phosphates were synthesized from a mixture of glucose and fructose (Table 6). With sucrose, esters were estimated by the difference method and also enzymically after hydrolysis of the reaction products with 0·1 N-HCl at 60° for 5 min. or after treatment with yeast invertase at pH 5·5 for 1 hr. at 20°. Non-enzymic synthesis of hexose phosphates (Morton, 1958*b*) was observed during the acid hydrolysis.

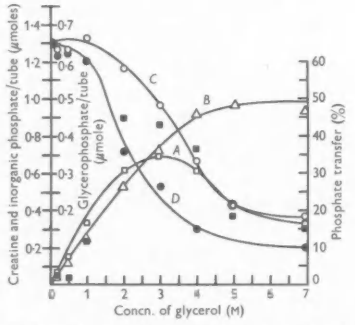

Fig. 5. Effect of glycerol concentration on the phosphotransferase reaction. Conditions were as for Fig. 3 except that the phosphocreatine concentration was 35 mM and glycerol replaced glucose. *A*, DL-Glycerophosphate calculated from enzymic estimation of L-α-glycerophosphate (□), or by difference between creatine and inorganic phosphate liberated (■); *B*, percentage transfer; *C*, creatine; *D*, inorganic phosphate.

Serine, threonine and ethanolamine. The reaction mixtures (0·4 ml.) contained 64 mM-$NaHCO_3$–Na_2CO_3 buffer, pH 9·2, mM-magnesium acetate, 2 μg. of intestinal alkaline phosphatase and either DL-serine (approx. M), DL-threonine (approx. 0·5 M) or ethanolamine (M). The reaction period was 30 min. at 38°. Spots of about 0·01 ml. were applied to Whatman paper no. 1 and ascending chromatograms were run in water-saturated

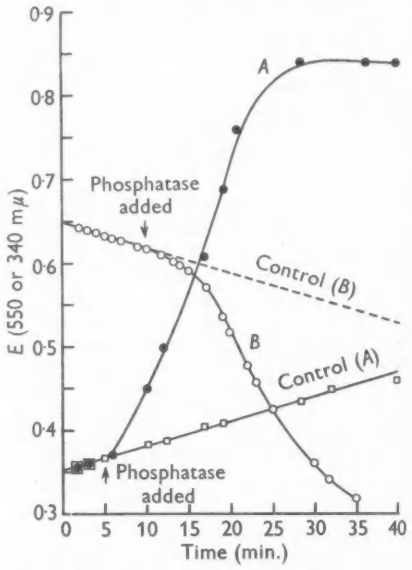

Fig. 6. Coupled reactions of glycerophosphate dehydrogenases and intestinal alkaline phosphatase. Curve *A* shows the reaction with cytochrome *c*-linked L-α-glycerophosphate dehydrogenase. Each cuvette (1 cm.) contained, in 2·6 ml., 2 M-glycerol, 0·01 M-$MgCl_2$, approx. 0·05 mM-ferricytochrome *c*; 0·05 M-veronal–sodium acetate–HCl buffer, pH 8·0, and 'particle-bound' L-α-glycerophosphate dehydrogenase of rabbit skeletal muscle. Cytochrome *c* was omitted from the blank cuvette. Readings were made at 550 mμ with a Beckman spectrophotometer model DU. After 4 min., 0·1 ml. of purified intestinal phosphatase was introduced. The reaction was carried out at room temperature (approx. 16°). Curve *B* shows the reaction with DPN-linked L-α-glycerophosphate dehydrogenase. Each cuvette (1 cm.) contained in 2·7 ml. 0·05 M-glycyglycine buffer, pH 8·2; 0·1 mM-reduced DPN; approx. M-DL-glyceraldehyde; 0·01 M-phenyl phosphate; an enzyme preparation from rabbit skeletal muscle containing both 'soluble' L-α-glycerophosphate dehydrogenase and triose phosphate isomerase. Readings were made at 340 mμ. After 10 min., 0·1 ml. of purified intestinal phosphatase was introduced. The reaction was carried out at room temperature (approx. 19°).

Table 4. *Phosphorylation of propane-1:2-diol catalysed by intestinal alkaline phosphatase*

The reaction mixture (0·5 ml.) contained 0·03 M-sodium bicarbonate–sodium carbonate buffer, pH 9·2; 35 mM-phosphocreatine; mM-magnesium acetate; 6 μg. of intestinal alkaline phosphatase; propane-1:2-diol at the concentrations indicated. The reaction period was 10 min. at 38°.

Concn. of propanediol (M)	Product/tube (μmoles)			Phosphate transfer (%)
	Inorganic phosphate	Creatine	Propanediol phosphate*	
0	2·40	2·38	—	—
1	1·96	2·38	0·42	18
3	1·31	2·04	0·73	36
5	0·93	1·60	0·67	42

* Difference between creatine and inorganic phosphate liberated.

Table 5. *Phosphorylation of glyceraldehyde and of dihydroxyacetone*

The reaction mixture (0·5 ml.) contained 0·05 M-glycine–NaOH buffer, pH 9·8; 0·08 M-phenyl phosphate; mM-magnesium acetate; approx. 1 μg. of intestinal alkaline phosphatase, and either DL-glyceraldehyde or dihydroxyacetone at the concentrations indicated. The reaction period was 10 min. at 38°.

Concn. of triose (M)		Products/tube (μmoles)		Phosphate transfer (%)
Glyceraldehyde	Dihydroxyacetone	Inorganic phosphate	Triose phosphate	
0	0	2·26	0	—
1	—	1·47	0·10	7
2	—	0·57	0·07	11
—	1	1·22	0·14	10
—	2	0·63	0·12	16
—	3	0·39	0·11	22

Table 6. *Results obtained with sucrose as an acceptor for phosphate in the transferase reaction with intestinal alkaline phosphatase*

The reaction mixture (0·5 ml.) contained 0·05 M-veronal–sodium carbonate buffer (King & Delory, 1940), pH 9·5; mM-magnesium acetate; 0·02 M-phosphocreatine; 9 μg. of intestinal phosphatase; sugars at the concentrations indicated. The reaction period was 5 min. at 38°.

Sugar	Concn. of sugar (M)	Products/tube (μmole)			
		Creatine	Inorganic phosphate	Sucrose phosphate† or hexose phosphate‡	Hexose phosphate‡
Sucrose	0	0·97	0·95	+0·02	—
	0·5	0·93	1·00	−0·07	—
	1	0·51	0·63	−0·12	0
	2	0·50	0·51	−0·01	0
	3	0·31	0·24	+0·07	—
Glucose–fructose mixture*	2	0·69	0·55	+0·14	0·05
	4	0·27	0·10	+0·17	0·05

* A mixture of equal weights of glucose and fructose was used, and treated identically as sucrose.
† Difference between creatine and inorganic phosphate formed.
‡ Determined enzymically, directly or after hydrolysis of the reaction products obtained with sucrose in 0·1 N-HCl at 60° for 5 min.

phenol (Dent, 1948). In each, the appearance of a new ninhydrin-positive spot in the reaction products of the tube containing the phosphatase, as compared with the control containing enzyme destroyed by boiling, was taken as presumptive evidence for the formation of some phosphorylated compound. However, no authentic marker compounds were available for comparison with the suspected phosphorylated component.

Nucleosides. Approx. 0·2 ml. of 0·05 M-ethanolamine–HCl buffer, pH 9·8, containing 0·02 M-β-glycerophosphate or 0·01 M-*p*-nitrophenyl phosphate was saturated with adenosine, guanosine, cytidine or uridine, and approx. 1 μg. of intestinal

alkaline phosphatase added. After 30 min. at 22°, 0·02 ml. was applied to Whatman no. 1 paper and dried immediately. Similar reaction mixtures containing authentic nucleotides were used as markers.

The esters were also precipitated as the barium salts, washed and converted into the ammonium salts. They were then applied to Whatman no. 1 paper as before. Descending chromatograms were run in the propan-1-ol–aq. NH_3 soln. solvent of Hanes & Isherwood (1949), and also in the saturated $(NH_4)_2SO_4$–sodium acetate–propan-2-ol solvent of Markham & Smith (1951). The chromatograms were examined by ultraviolet light (Markham & Smith, 1949).

A very small amount of adenosine monophosphate was detected, but, apart from adenosine, no other nucleoside was phosphorylated.

Donor specificity in the transferase reaction

Table 7 shows that a number of the typical substrates of both intestinal and milk phosphatases act as donors in the transfer reaction.

With phenyl phosphate (0·01 M) and glucose (2 M) at pH 10·2, similar percentage transfers were obtained with a preparation of microsomes from calf intestinal mucosa (Morton, 1954), and with partially purified and purified preparations of intestinal alkaline phosphatase.

Comparative studies with substrate-specific phosphatases and alkaline phosphomonoesterases

Kidney hexose diphosphatase and milk alkaline phosphatase. Glycerol was used as acceptor, and hexose diphosphate as phosphate donor. Synthesis of L-α-glycerophosphate was measured enzymically with cytochrome *c*-linked α-glycerophosphate dehydrogenase (Morton, 1958*a*). Whereas there was no detectable synthesis of L-α-glycerophosphate with kidney hexose diphosphatase there was considerable synthesis by milk alkaline phosphatase under similar conditions (Table 8). Under the assay conditions used, 0·02 μmole of DL-α-glycerophosphate/tube would be estimated.

5′-Nucleotidase and intestinal alkaline phosphatase. The reaction mixture (0·5 ml.) contained

Table 7. *Phosphate transfers from various donor compounds*

Acceptor	Donor	Free energy of hydrolysis ($-\Delta F'$) of donor† (kcal./mole)	Reaction conditions: Concn. of donor (M)	pH	Phosphate transfer (%) at acceptor concn.* (M): 1	2	3	4	5
Glucose	Creatine phosphate	10·5	0·026	9·8	12	14	14	16	—
	Phenolphthalein phosphate	—	0·01	9·8	—	14	—	—	—
	Phenyl phosphate	—	0·01	10·0	10	16	—	14	—
	Adenosine 5-phosphate	—	0·005	9·0	—	10	—	—	—
	β-Glycerol phosphate	2·1‡	0·02	9·6	—	16	—	14	—
Glycerol	Creatine phosphate	10·5	0·035	9·8	14	18	33	45	45
	Phospho-enol pyruvate	12·8	0·002	9·6	—	—	—	43	—
	Hexose diphosphate	—	0·005	9·5	—	14	—	43	—
	β-Glycerol phosphate	2·1‡	0·02	9·8	—	18	—	40	—
	p-Nitrophenyl phosphate	—	0·01	9·8	—	20	—	—	—

* Based on enzymic estimations of glucose phosphate and α-glycerophosphate.
† Values are given for pH 7 and 25°, as estimated by Atkinson & Morton (1958); see also Oesper (1951).
‡ Value for α-glycerophosphate.

Table 8. *Comparison of reactions catalysed by milk alkaline phosphatase and kidney hexose diphosphatase*

The reaction mixture (0·5 ml.) contained 0·05 M-veronal–sodium carbonate buffer, pH 9·5; 0·01 M-magnesium acetate; 5 mM-fructose 1:6-diphosphate, glycerol at the concentrations indicated; either hexose diphosphatase (approx. 0·3 mg.) or milk alkaline phosphatase (approx. 10 μg.). The reaction period was 10 min. at 38°.

Concn. of glycerol (M)	Products/tube (μmole): Hexose diphosphatase: Inorganic phosphate	Hexose diphosphatase: α-Glycerol phosphate	Alkaline phosphatase: Inorganic phosphate	Alkaline phosphatase: α-Glycerol phosphate	Phosphate* transfer (%) with alkaline phosphatase
0	1·32	—	1·29	—	—
1	1·39	0	—	—	—
2	1·26	0	0·68	0·11	14
3	1·16	0	—	—	—
4	1·23	0	0·38	0·19	33
5	1·18	0	—	—	—

* Assuming only DL-α-glycerophosphate is formed.

5 mM-AMP, 2 M-glucose, 0·01 M-$MgCl_2$, 0·02 M veronal–HCl buffer, pH 9·0, and 0·1 ml. of either 5′-nucleotidase or intestinal phosphatase. The incubation time was 10 min. at 38°, the amount of enzyme being adjusted to liberate about 1·1 μmoles of inorganic phosphate (about 44 % hydrolysis of the substrate) in each case.

No glucose phosphate was detected in a 1 ml. portion of the reaction products of 5′-nucleotidase so that less than 0·02 μmole was formed, whereas 0·12 μmole was synthesized by the intestinal phosphatase (11 % transfer).

Myosin–adenosine triphosphatase and intestinal alkaline phosphatase. The reaction mixtures (0·5 ml.) contained 0·02 M-veronal–HCl buffer, pH 8·5, 0·01 M-$CaCl_2$, 25 mM-adenosine triphosphate (ATP), glucose at 0, 2 and 4 M concentrations and sufficient myosin preparation (0·1 ml.) to hydrolyse about 55 % of the substrate in the incubation time (30 min.) at 20°. No glucose phosphate was detected in any tube, so that the amount formed was less than 0·02 μmole. The amount of inorganic phosphate liberated at 4 M-glucose (0·61 μmole) was little different from that in the absence of glucose (0·69 μmole). With intestinal phosphatase (approx. 20 μg.) under similar conditions (except that $MgCl_2$ replaced $CaCl_2$) and also at pH 9 with 5 mM-ATP, mM-magnesium acetate and 1, 2 and 4 M-glucose, there was no significant hydrolysis of ATP and no synthesis of glucose phosphate.

DISCUSSION

The two alkaline phosphatases used in this investigation were from different sources and were highly purified (Morton, 1953*a*, 1954). Although they have similar substrate specificity, they are quite different enzymes (Morton, 1955*a*). Under similar experimental conditions, the efficiency of phosphorylation of glucose by microsomes from intestinal mucosa was the same as that obtained with partially purified and purified enzyme preparations. With the purified enzymes from intestinal mucosa and from milk, the initial rates of phosphate transfer and of hydrolysis were similarly influenced by the pH, and by the concentration of phosphate donor or inorganic phosphate. These findings establish that the phosphotransferase activity is undoubtedly a property of the mucosal and milk alkaline phosphatases themselves.

Table 1 confirms the synthesis of esters from inorganic phosphate and sugars (Kay, 1928; Meyerhof & Green, 1949). Large amounts of inorganic phosphate inhibit both the hydrolytic and the transferase activities of alkaline phosphatase (cf. Tables 1 and 2). Experimental conditions may be chosen so that ester phosphate is synthesized from phosphocreatine and glucose, but not from comparable (or smaller) concentrations of inorganic phosphate and glucose (Table 2). It is clear therefore that the transferase reaction of alkaline phosphatase involves direct transfer of the phosphate group from the donor without the intermediary formation of inorganic phosphate, a finding previously established by the use of radioactive phosphorus (Meyerhof & Green, 1950).

Meyerhof & Green (1950) suggested that both the rate of synthesis of new ester and the efficiency of phosphorylation (percentage transfer) are determined by the free energy of hydrolysis of the donor. These workers used as phosphate donors phosphocreatine and glucose 1-phosphate, and in later work (Green & Meyerhof, 1952) acetyl phosphate, phosphocreatine, phosphopyruvate and *p*-nitrophenyl phosphate. As already pointed out (Morton, 1952*a*, *b*; 1953*b*), the rates of synthesis of glucose and glycerol phosphates are not related to the free energy of hydrolysis of the donor, but rather are determined primarily by the affinity of the enzyme for the particular donor used (see Table 7). Under comparable conditions of pH, enzyme and acceptor concentrations, the donors which are the more rapidly hydrolysed also give the higher rates of formation of new ester. Compounds (such as ATP) which are not hydrolysed (see above) do not act as donors for the transferase reaction. With the one acceptor at a given concentration, substantially the same percentage transfer was achieved with all substrates investigated, which included β-glycerophosphate and phosphocreatine (Morton, 1953*b*, and this paper, Table 7). The same is true of the prostate acid phosphatase also (Morton, 1953*b*, 1958*b*). The percentage transfer is chiefly a function of the affinity of the enzyme for the acceptor, and the acceptor concentration. The results indicate the importance of the enzyme complexes involved in the reaction and suggest that the activation energies for the enzymic reaction, rather than the free energy of hydrolysis of the donor, determine the rate of synthesis of new ester and probably also the percentage transfer.

Although both the intestinal and milk alkaline phosphatases, and prostate acid phosphatase (Appleyard, 1948; Morton, 1952*a*, 1953*b*, 1958*b*), catalyse simultaneous phosphate-transfer and hydrolysis reactions, it cannot be concluded that this is a general property of phosphatases (Meyerhof & Green, 1950). The results with hexose diphosphatase, 5′-nucleotidase and myosin–adenosine triphosphatase suggest that these enzymes catalyse hydrolysis only. Although a far wider range of acceptors should be examined in order to substantiate this finding, the maintenance of the rate of hydrolysis in the presence of high concentrations of acceptors, and hence lowered concentrations of free water, supports this view (Table 5, and Morton,

1955*c*). The failure of myosin–adenosine triphosphatase to catalyse phosphate-exchange reactions (Koshland, Budenstein & Kowalsky, 1954; Koshland, 1955) further substantiates the absence of phosphotransferase activity (Morton, 1953*b*). Heppel & Hilmoe (1951) reported that the specific yeast inorganic pyrophosphatase fails to catalyse synthesis of organic pyrophosphates. Thus phosphatases may be classified into two groups, namely (*a*) those enzymes which are specific hydrolases, in that water is the only phosphate acceptor, and (*b*) those which are transferases as well as hydrolases, in that other hydroxy compounds in addition to water may act as phosphate acceptors (Morton, 1952*a*, *c*; 1953*b*).

Acceptor specificity of alkaline phosphatases

The results show that the polyols and trioses used in this study are more effective than hexoses or sucroses at similar acceptor concentrations. These compounds do not contain ionizable groups, and where these are present the percentage transfer may vary with pH, whereas it is independent of pH with glucose and similar acceptors (Fig. 2). The influence of groups adjacent to the hydroxyl, and of the configuration and size of the acceptor, probably accounts for the absence of appreciable transfer to sucrose, to the nucleosides or to diphosphopyridine nucleotide (Morton, 1955*d*). The preliminary findings of phosphorylation of serine, threonine and ethanolamine require confirmation.

These results indicate that the various acceptors, including water, are absorbed at specific sites of the enzyme surface (Morton, 1952*a–c*, 1953*b*, 1955*c*). Whereas the qualitative specificity of the two alkaline phosphatases towards hydroxy compounds is similar, there may be quantitative differences, as suggested by the lower phosphorylation of glucose and of glycerol by the milk enzyme (cf. Table 3 and Fig. 3, and Table 8 and Fig. 5).

The specificity of the alkaline phosphatases to the products synthesized is especially noteworthy. The results show that DL-α-glycerophosphate is the major product formed by phosphate transfer to glycerol, whether β-glycerophosphate or another compound is the donor. Whereas both α- and β-glycerophosphates are readily hydrolysed by alkaline phosphatases (Morton, 1955*b*), α-glycerophosphate is the major product formed from inorganic phosphate and glycerol (Meyerhof & Green, 1949). The synthesis of α-glycerophosphate from β-glycerophosphate (Table 7) ostensibly is a 'phosphoglyceromutase' reaction (see Dixon & Webb, 1953). Glucose 6-phosphate is the major product formed in the transferase reaction with glucose, as it is also in the synthesis from inorganic phosphate and glucose (Meyerhof & Green, 1949, and Table 1). Surprisingly, the predominant initial product formed with fructose seems to be fructose 6-phosphate with a small amount of fructose 1:6-diphosphate (see Results section), although most of the sugar would be in the pyranose configuration and so would be expected to give fructose 1-phosphate. The results mentioned above (and Fig. 4) suggest that fructose in the furanose configuration is preferentially phosphorylated. Since, under the experimental conditions, fructofuranose is about 20–30% of the total fructose (Gottschalk, 1945) this may account for the much lower rate of phosphorylation found with fructose (Fig. 4) as compared with glucose (Fig. 3). Meyerhof & Green (1949), however, found that the product of inorganic phosphate and fructose incubated with phosphatase for 72 hr. at 38° was about 65% of fructose 1-phosphate and 28% of fructose 6-phosphate, as well as a small amount of fructose 1:6-diphosphate.

Mechanism of the phosphotransferase reaction

The plots of initial reaction rates at increasing concentrations of acceptors (Figs. 3–5) provide information concerning the mechanism of the transferase reactions of hydrolytic enzymes (Morton, 1952*a–c*, 1953*b*, 1955*c*). In each, the rate of synthesis of new ester increases with increasing concentration of acceptor up to a limiting concentration, beyond which further increase of acceptor concentration causes a fall in the rate of synthesis. The rate of hydrolysis decreases with increase of the acceptor concentration. The percentage transfer increases with acceptor concentration up to a maximum value, which occurs close to the concentration of acceptor which gives the maximum rate of ester synthesis. Further increase of acceptor concentration causes no further change in the percentage transfer.

The plot of synthesis rate versus acceptor concentration (Fig. 3, curve *C*, for example) is of a form resembling that obtained with many enzymes for the plot of rate of formation of product versus substrate concentration. It is consistent with the view that the acceptor is adsorbed at a specific site of the enzyme. It is assumed that this site is saturated when the acceptor reaches the limiting concentration giving the maximum rate of ester synthesis. From Figs. 3–5, and from Table 4, the concentrations of acceptors giving half the maximum rates of synthesis are approx. 0·4, 0·4, 0·9 and 0·9M for glucose, fructose, glycerol and propane-1:2-diol respectively.

The decline in the rate of hydrolysis with increasing concentration of acceptor (Figs. 3–5) may be due to the lowering of the concentration of water molecules and also to direct competition of the other hydroxy compound with the water. The fall in the rates of synthesis and of hydrolysis at very

high concentrations of acceptors (e.g., between 2 and 4M-glucose in Fig. 3) appears to be due to unspecific effects of low water content.

These observations suggest that the transferase activity is the result of the competition between the acceptor molecules and water molecules for sites at the enzyme surface. The two may not necessarily compete for the same site (Morton, 1955*c*), although this is not unlikely. The interaction of acceptor molecules with the enzyme–donor complex (or its reaction product) in some cases may lead to an increased rate of utilization of the donor as compared with the rate in the absence of added acceptor (see Fig. 3, curve *A*, for example.)

A direct non-enzymic transfer of phosphate from phosphocreatine to glucose may also occur under acid conditions (Morton, 1952*a*, *b*, 1958*b*). The difference between the products formed in this reaction and those formed by phosphotransferase action supports the suggestion that the acceptor is adsorbed at the enzyme surface. A mechanism for the transferase action, which is consistent with these kinetic results, has been proposed (Morton, 1952*c*, 1953*b*, 1955*c*). However, it is recognized that kinetic studies, by themselves, cannot establish any reaction mechanism, and the suggested sequence of reactions is by no means proven. Alternative interpretations of the phosphotransferase reaction have been offered (Koshland, 1955; Swoboda, 1955). Formulation of any reaction mechanism for the alkaline phosphatases must take into account not only the kinetic behaviour in respect of the transferase reaction, but also the kinetics of hydrolysis (Morton, 1957).

SUMMARY

1. Alkaline phosphatases from calf intestinal mucosa and from cow's milk catalyse synthesis of phosphate esters by direct transfer of the phosphate group from phosphomonoesters to suitable hydroxy compounds. There is no requirement for inorganic phosphate.

2. Kinetic studies of the transfer reaction were carried out with glucose, fructose and glycerol as phosphate acceptors. The products were estimated enzymically.

3. Over the range pH 8–10, the rate of synthesis of glucose phosphate varied with pH, but the percentage transfer was independent of pH. The rate of synthesis of new ester and of hydrolysis of the donor varied with the nature and concentration of the donor, but the percentage transfer was independent.

4. Whereas the rate of hydrolysis of the donor decreases, the rate of synthesis of new ester, and of percentage transfer, increases with acceptor concentration up to a limiting value which varies with each acceptor.

5. Neither the initial rate of synthesis of new ester nor the percentage transfer is related to the free energy of hydrolysis of the donor, but both vary considerably with the nature of the acceptor.

6. Kidney hexose diphosphatase, 5′-nucleotidase and myosin–adenosine triphosphatase failed to catalyse transfer to acceptors other than water.

7. Competition between water and other hydroxyl-containing compounds for sites at the surface of the enzyme–donor complex is postulated to explain the reactions of the alkaline phosphomonoesterases.

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The Phosphotransferase Activity of Phosphatases

3. COMPARISON OF ENZYMIC CATALYSIS BY ACID PHOSPHATASE WITH NON-ENZYMIC CATALYSIS AT ACID pH VALUES

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In a preceding paper (Morton, 1958*a*) it was shown that highly purified alkaline phosphomonoesterases from cow's milk and from calf intestinal mucosa have associated 'phosphotransferase' (Dixon, 1949; Dixon & Webb, 1953) activity. The kinetics of the phosphorylation of glucose, fructose, glyceraldehyde, dihydroxyacetone and glycerol were investigated with sensitive enzymic methods developed for estimation of the phosphorylated products (Morton, 1958*b*).

This paper describes similar studies with a partially purified acid phosphatase from prostate gland, the phosphotransferase activity of which was first observed by Appleyard (1948). During this investigation it was found that glucose phosphates were formed by phosphate transfer from phosphocreatine in a non-enzymic reaction occurring at the same pH values as used for the studies with the acid phosphatase. A comparison was made of the enzymic and non-enzymic reactions (Morton, 1952*a*).

This work has been briefly reported elsewhere (Morton, 1952*b*, 1953).

MATERIALS AND METHODS

Except for the acid-phosphatase preparation, the materials and methods have been described in a preceding paper (Morton, 1958*a*).

Acid phosphatase. A human prostate gland was finely minced and then ground to a smooth paste in a mortar with fine acid-washed sand and 0·15 M-NaCl, pH 7·0 (at 22°). The paste was diluted with 5 vol. of 0·15 M-NaCl and the suspension stirred gently for 30 min. and then centrifuged at 2000 *g* for 30 min. The supernatant was then re-centrifuged at 14 000 *g* for 30 min. The clear supernatant was fractionated by addition of $(NH_4)_2SO_4$, the pH being maintained at about 6·8 at 20°. The precipitate obtained between 55 and 65% saturation with $(NH_4)_2SO_4$ was dissolved in water and dialysed in seamless cellophane tubing against water for 24 hr. at 0–4°. A precipitate was removed by centrifuging at 14 000 *g* for 30 min. in a cold-room at 2°, and the clear supernatant was stored at 0°.

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RESULTS

Reactions catalysed by acid phosphatase

Phosphorylation of glucose. The reaction mixtures contained acid phosphatase (except in the control tube), 8 mM-phosphocreatine, 0·1 M-sodium acetate buffer, pH 5·5, and increasing concentrations of glucose up to 4 M. All components except the phosphocreatine were added to the tubes, the volumes were adjusted to 0·4 ml. and the tubes brought to 38°. The reaction was commenced by addition of phosphocreatine, and stopped after 5 min. at 38° by addition of trichloroacetic acid as already described (Morton, 1958*b*). The analyses were carried out as described before (Morton, 1958*a*, *b*), the glucose phosphate being estimated enzymically. Fig. 1 shows that the rate of synthesis of glucose phosphate (curve *C*) increased continuously up to 3·5 M-concentration of glucose. The rate of hydrolysis, as indicated by the liberation of inorganic phosphate (curve *B*), declined continuously with increasing concentrations of glucose, whereas the rate of utilization of phosphocreatine (due to both the hydrolysis and transfer reactions) remained fairly constant (curve *A*). Curve *D* shows that the percentage transfer (see Morton, 1958*a*) increased to about 13% with 4 M-glucose.

Table 1. *Phosphorylation of glycerol and propane-1:2-diol catalysed by acid phosphatase*

The reaction conditions for phosphorylation of propane-1:2-diol were similar to those for Fig. 1, except that the polyol was substituted for glucose. For the experiment with glycerol, 0·02 M-phenyl phosphate replaced the phosphocreatine.

Acceptor	Concn. of acceptor (M)	Product/tube (μmoles) Creatine or phenol	Inorganic phosphate	Polyol* phosphate	Phosphate transfer (%)
1:2-Propanediol	0	1·25	1·27	—	—
	3	1·47	0·68	0·79	54
	5	0·55	0·25	0·30	55
Glycerol	0	3·75	3·75	—	—
	3	5·91	2·40	3·51	60

* Difference between creatine or phenol, and inorganic phosphate.

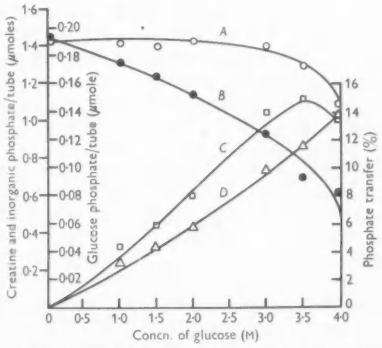

Fig. 1. Phosphorylation of glucose catalysed by acid phosphatase. The reaction mixture (0·5 ml.) contained 0·1 M-sodium acetate buffer, pH 5·5; 8 mM-phosphocreatine; glucose; 0·05 ml. of acid phosphatase. The reaction was commenced by addition of phosphocreatine and was carried out for 5 min. at 38°. *A*, Creatine; *B*, inorganic phosphate; *C*, glucose phosphate determined enzymically; *D*, percentage transfer.

Table 2. *Phosphorylation of glyceraldehyde and of dihydroxyacetone catalysed by acid phosphatase*

Reaction conditions were similar to those for Fig. 1 except that the trioses replaced glucose.

Concn. of acceptor (M) DL-Glyceraldehyde	Dihydroxyacetone	Product/tube (μmole) Inorganic phosphate	Triose phosphate
0	0	1·81	—
1	—	1·72	0·26
—	1	1·70	0·31
—	2	1·63	0·59

With phenyl phosphate (0·01 M) or β-glycerophosphate (0·01 M) as donor and with 4 M-glucose at pH 5·5 as above, the percentage transfer was similar (approx. 14%).

Phosphorylation of glycerol and propane-1:2-diol. Table 1 shows that both polyols are phosphorylated.

Phosphorylation of glyceraldehyde and dihydroxyacetone. Both these trioses are phosphorylated. Table 2 shows the influence of the concentration of the trioses on the phosphotransferase reaction.

Phosphorylation of nucleosides. The reaction mixture (0·2 ml.) contained 0·05 M-*p*-nitrophenyl phosphate, 0·1 M-sodium acetate buffer, pH 5·2 at 20°, 0·1 M-cytidine and phosphatase. About 4 μmoles of inorganic phosphate were liberated during the reaction (30 min. at 20°). Paper chromatograms were prepared and examined as previously described (Morton, 1958*a*). Papers run in the propan-1-ol–aq. NH_3 soln. solvent of Hanes & Isherwood (1949) showed two new but rather faint spots in the products formed with acid phosphatase. One of these ran in an identical position to authentic cytidine 5′-phosphate. The other spot was probably cytidine 3′-phosphate or cytidine 2′-phosphate, or a mixture of both.

Non-enzymic phosphate transfer

Phosphorylation of glucose. During the investigation of the acceptor specificity of alkaline phosphatase, a mixture containing sucrose (2 M) and phosphocreatine (13 mM) was hydrolysed with 0·1 N-HCl at 60° for 5 min. Determination of glucose phosphate enzymically showed large amounts to be present both in the 'control' and the 'test' (which had been incubated with alkaline phosphatase; see Morton, 1958*a*). The formation of glucose phosphate during acid hydrolysis of phosphocreatine was therefore investigated.

The reaction mixture contained glucose, sodium acetate buffer and phosphocreatine. The reaction was commenced by addition of phosphocreatine and was carried out at pH 4·2 for 10 min. at 38°. Glucose phosphate was estimated enzymically and by difference between the creatine and inorganic phosphate liberated.

Table 3. *Non-enzymic synthesis of glucose phosphates*

In Expt. *A*, the reaction mixture (0·5 ml.) contained 0·2 M-sodium acetate buffer, pH 5·8, 0·5% ammonium molybdate, 26 mM-phosphocreatine and glucose. The reaction was commenced by addition of phosphocreatine and was continued for 5 min. at 38°.

In Expt. *B*, the reaction mixture (0·5 ml.) contained 26 mM-phosphocreatine and M-glucose in 0·1 N-HCl. The reaction was carried out for 7 min. at 100°.

Glucose phosphate was estimated enzymically.

Experiment	Concn. of glucose (M)	Products/tube (μmoles) Creatine	Inorganic phosphate	Glucose phosphate	Phosphate transfer (%)
A	0	2·00	1·96	—	—
	2	1·91	1·65	0·08	4
	4	1·93	1·47	0·14	7
B	1	13·2	11·9	0·59	4

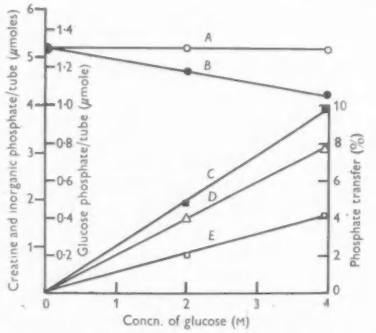

Fig. 2. Non-enzymic synthesis of glucose phosphate. The reaction mixture (0·5 ml.) contained 0·1 M-sodium acetate buffer, pH 4·2; 16 mM-phosphocreatine; glucose. The reaction was commenced by addition of phosphocreatine, and was carried out for 10 min. at 38°. *A*, Creatine; *B*, inorganic phosphate; *C*, glucose phosphate as estimated from the difference between creatine and inorganic phosphate; *D*, percentage transfer; *E*, glucose phosphate, estimated enzymically.

Table 4. *Non-enzymic phosphorylation of dihydroxyacetone*

The reaction mixture (0·5 ml.) contained 0·2 M-sodium acetate buffer, pH 4·2, 26 mM-phosphocreatine and dihydroxyacetone. The reaction was commenced by the addition of phosphocreatine and was continued for 5 min. at 38°.

Concn. of dihydroxyacetone (M)	Product/tube (μmoles) Inorganic phosphate	Triose phosphate
0	5·02	—
1	4·29	0·48
2	3·47	0·89

Fig. 2 shows that the rate of formation of both total glucose phosphate (curve *C*) and of enzymically estimated glucose phosphate (curve *E*) increases linearly with glucose concentration. The rate of hydrolysis (as indicated by curve *B*) declines linearly with increasing concentration of glucose, so that the rate of creatine liberation (curve *A*) remains constant. The percentage transfer based on enzymically estimated glucose phosphate (curve *D*) increases continuously up to the maximum concentration of glucose.

Table 3 shows that essentially similar results were obtained at pH 5·8 with 0·5% ammonium molybdate in the reaction mixture.This was added as it was thought that it catalysed the hydrolysis of phosphocreatine. The compound, however, is not essential for the reaction. Table 3 also shows that glucose phosphates are formed from phosphocreatine and glucose at 100° in 0·1 N-HCl.

Phosphorylation of dihydroxyacetone. Table 4 shows that the rate of synthesis of dihydroxyacetone phosphate (as estimated enzymically) increases with the acceptor concentration.

DISCUSSION

Appleyard (1948) used a crude extract of prostate gland with phenolphthalein phosphate as donor and a number of aliphatic alcohols and glycols as acceptors. The discrepancy between the phenolphthalein and the inorganic phosphate found in the reaction products was attributed to the activity of a 'transphosphorylase'. However, comparison of the kinetic behaviour of the acid phosphatase with glucose, trioses and polyols as acceptors with that of the purified alkaline phosphatases (Morton, 1958*a*) with similar acceptors indicates that the transferase activity is a property of the phosphatase itself. In crude and highly purified preparations of prostate-gland phosphatase the ratio of transferase to hydrolase activity is the same, and the heat-denaturation constants for the two activities are similar (London & Hudson, 1955). Axelrod (1948*a*) established that inorganic phosphate is not an intermediate in the phosphate transfer catalysed by acid phosphatases.

Like the alkaline phosphatases (Morton, 1955*a*), prostate-gland acid phosphatase has a wide substrate specificity (Delory, Wiberg & Hetherington, 1955) and hydrolyses compounds having an N–P linkage, such as phosphocreatine (Morton, 1952*a*, 1955*a*; Møller, 1955), as well as typical orthophosphomonoesters. The rate of hydrolysis of phosphocreatine by this enzyme is markedly dependent on the substrate concentration. Relative initial rates of hydrolysis were 100 and 20 μg. of P liberated/min./0·1 ml. of enzyme at 5 and 50 mM-phosphocreatine respectively. Hence the choice of the relatively low (8 mM) substrate concentration in the transferase studies (Fig. 1).

Although the kinetic results presented here are limited as compared with those obtained with alkaline phosphatases (Morton, 1958*a*), certain similarities in the transferase activities of the acid and alkaline phosphatases are apparent. With three donors (phenyl phosphate, glycerophosphate and phosphocreatine), the transferase activity was parallel with hydrolytic activity, and the percentage transfer with 4M-glucose was essentially the same. Thus the efficiency of phosphorylation is independent of the standard free energy of hydrolysis of the donor (Morton, 1953). With two concentrations (8 and 50 mM) of phosphocreatine, the same percentage transfer to 2M-glucose was obtained, although very much less glucose phosphate was formed at the higher substrate concentration, owing to inhibition of the enzyme (see above). Tsuboi & Hudson (1953) found that the acid phosphatase of haemolysed human red cells catalysed transfers from phenyl phosphate to glycerol and methanol, and that the ratio of phosphate transferred to phosphate hydrolysed was independent of the concentration of phenyl phosphate. Axelrod (1948*b*) reported a similar finding with citrus-fruit acid phosphatase.

As with intestinal alkaline phosphatase (Morton, 1958*a*), so with prostate acid phosphatase, the relative distribution of the transferred phosphate groups among the water and acceptor molecules varies with the concentration of acceptor (Fig. 1 and Tables 1 and 2). However, the rate of glucose phosphate synthesis increases almost linearly with increasing concentration of glucose up to between 3·5 and 4M, whereas with alkaline phosphatase a well-defined maximum appears at between 1·5 and 2·0M-glucose (cf. this paper, Fig. 1, and Morton, 1958*a*, fig. 3). The percentage transfer also increases up to the maximum concentration of glucose (Fig. 1).

With the trioses (Table 2) and polyols (Table 1) as phosphate acceptors, the rate of utilization of the donor is higher as compared with the rate in the absence of acceptor, as also observed with acid phosphatases from human red cells (Tsuboi & Hudson, 1953) and from citrus fruits (Axelrod, 1948*b*).

Acceptor specificity of phosphatases and nature of synthesized products

The studies reported here extend and supplement those of Appleyard (1948). The two investigations show that the prostate-gland acid phosphatase has a wide acceptor specificity. The percentage transfer to these different acceptors varies according to the nature of the acceptor, as was shown for citrus-fruit acid phosphatases by Axelrod (1948*b*) and for two alkaline phosphatases by Morton (1958*a*).

As with alkaline phosphatase (Morton, 1958*a*), so with the prostate enzyme, the newly formed ester probably accumulates because of the relatively low rate of hydrolysis of the newly formed ester as compared with that of the donor compound. The optimum pH of hydrolysis of esters by prostate acid phosphatase varies with the nature of the ester (Delory *et al.* 1955; Lundquist, 1947). Conditions chosen for optimum rate of utilization of the donor will generally be grossly suboptimum for hydrolysis of the newly formed ester. With the usual reaction conditions, at equilibrium there would be negligible amounts of donor compound or of newly formed ester.

With intestinal alkaline phosphatase (Morton, 1958*a*) the enzymically estimated glucose phosphates accounted for between 80 and 100% of the phosphate which was apparently transferred to glucose, and DL-α-glycerophosphate for most of the phosphate which was apparently transferred to glycerol. With prostate-gland acid phosphatase, however, in different experiments the enzymically estimated glucose phosphate was between 30 and 65% of the total phosphate apparently transferred to glucose, and, in one experiment, DL-α-glycerophosphate was about 60% of the phosphate apparently transferred to glycerol. Brawerman & Chargaff (1954*a*) found that about equal amounts of 5′- and 3′-cytidylic acid, and less of the 2′-cytidylic acid, were formed with cytidine. As previously pointed out (Morton, 1955*b*), these results indicate that prostate acid phosphatase is less specific in the position of phosphorylation of sugars and polyols than intestinal alkaline phosphatase.

Non-enzymic phosphorylation at acid pH *values*

The finding of a non-enzymic phosphate transfer from phosphocreatine to glucose (Morton, 1952*a*, *b*) was entirely unexpected. The reaction proceeds simultaneously with hydrolysis of this acid-labile compound, and occurs under similar conditions to those used with prostate acid phosphatase, namely at pH 5·8 and 38° (Table 3). Here the catalysis can be attributed only to H^+ or OH^- ions, and no

surfaces are involved. Hence the linear relationships for the rate of synthesis of glucose phosphate (Fig. 2, curve *B*) and rate of hydrolysis (curve *C*) with increase of glucose concentration is to be expected. The results indicate a direct competition of glucose molecules with water molecules, since the rate of liberation of creatine (curve *D*) remains quite constant at the different glucose concentrations.

Table 3 indicates a possible source of error in the estimation of phosphocreatine as 'acid-labile phosphate'. If the reaction mixture also contains glucose (or other suitable hydroxy compounds) under the usual conditions for estimation of 'acid-labile phosphate' (digestion for 7 min. at 100° in 0·1 N-HCl), some of the phosphate will be transferred from phosphocreatine to glucose and will fail to appear as inorganic phosphate. Under the conditions used in Table 3 (M-glucose and 26 mM-phosphocreatine) approx. 10% of the phosphate was transferred to glucose and approx. 4% appeared as glucose 6-phosphate.

Only 25–40% of the phosphate apparently transferred to glucose is estimated by the enzymic method of Slater (1953), indicating the synthesis of other esters in addition to glucose 1- and 6-phosphate. A negligible amount of the acid-labile glycosidic ester would be expected under the reaction conditions. Seegmiller & Horecker (1951) found that all hydroxyls of glucose except that of C-1 were phosphorylated by polyphosphoric acid, and the results suggest that the acid-catalysed transfer from phosphocreatine may also produce random phosphorylation. With dihydroxyacetone, for example, some triose diphosphate may be synthesized as well as the monophosphate, but only the latter would be estimated enzymically (Table 4). It is probable that a wide variety of other hydroxyl-containing compounds would act as phosphate acceptors in this non-enzymic-transfer reaction. The synthesis of histidine phosphate by non-enzymic transfer from amino phosphate was recently reported by Müller, Rathlev & Rosenberg (1956).

Biological significance of the phosphotransferase reaction

The variation in activity of alkaline phosphatase during growth and development of several organs indicates that this type of enzyme is associated with growth processes (see reviews by Moog, 1946, and by Bradfield, 1949). Reactions with compounds concerned in the metabolism of nucleic acids or of proteins could explain this relationship. Morton (1952*a*, 1953) showed that nucleotides could act as phosphate donors for the phosphotransferases, and Brawerman & Chargaff (1954*a*, *b*) that various nucleosides could act as phosphate acceptors. These latter workers used the name 'nucleoside transferases' for enzymes catalysing nucleoside phosphorylation. Their results are complicated by the use of crude tissue extracts and long incubation periods (17–30 hr. at 30°). Tunis & Chargaff (1956) have used adsorption techniques to separate hydrolytic and transferase activities of a carrot extract which catalyses phosphate transfer from phenyl phosphate. Plant extracts contain a number of so-called 'isodynamic' acid phosphatases (see Roche, 1950) which may be separated by adsorption methods (Boroughs, 1954). These may well differ quantitatively in acceptor specificity, and this could explain the results of Tunis & Chargaff (1956).

Whether the phosphomonoesterases function *in vivo* as transferases, or as hydrolases, may depend on the concentration of available water. The increased rate of utilization of the phosphate donor and the decreased rate of hydrolysis by acid phosphatases with increasing concentrations of suitable phosphate acceptors is in sharp contrast with the behaviour of the substrate-specific phosphatases (Morton, 1953, 1955*b*). The alkaline phosphatases of calf intestine and of cow mammary gland are probably associated with the lipoprotein structure of the endoplasmic reticulum, which gives rise to microsomes on cell rupture (Morton, 1954*a*, *b*; Hodge & Morton, 1956; Hodge *et al.* 1956). Liver acid phosphatase is bound to lipoprotein particles called lyosomes (Appelmans, Wattiaux & de Duve, 1955). The association of these enzymes with lipoprotein structures may restrict hydrolysis and favour transferase action *in vivo* (Morton, 1952*a*). However, in view of the wide specificity towards donors and acceptors shown by both the acid and alkaline phosphatases, the nature of the substrates of these enzymes *in vivo* is a matter for speculation at the present time.

SUMMARY

1. The transferase reaction catalysed by acid phosphatase of human prostate gland was studied in relation to the specificity towards donors and acceptors, and the influence of the concentration of donor and acceptor on the transfer reaction.

2. Non-enzymic phosphorylation of glucose with phosphocreatine as donor occurs at pH 4·2 and at 38°.

3. The results indicate that certain hydroxyl-containing compounds can compete with water in both the enzymic- and non-enzymic-transfer reactions. The possible biological significance of the transferase activity of phosphatases is discussed.

I wish to thank Dr M. Dixon, F.R.S., for his stimulating interest and advice in this work, which was carried out in

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Cell-wall Polysaccharides of Myrobalans

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Myrobalans are the dried fruit of an Indian tree, *Terminalia chebula* Retz.; they contain 43–53 % of tannin.

The present study was undertaken as there was no information relating to the cell-wall components, particularly as this is the first combretaceous plant to be investigated in this way. However, since the present work was carried out with dried-plant material, changes may have occurred in the polysaccharides during drying (cf. the rapid depletion of hemicelluloses in ripening banana; Barnell, 1943). This limitation applies with less emphasis to such polysaccharides as cellulose and polygalacturonic acid, at least some proportion of which would be expected to survive the changes occurring during the rapid drying process.

MATERIALS AND METHODS

Paper chromatography was effected in an all-glass apparatus at a constant temperature of 25°. Chromatograms were dried at room temperature, unless otherwise stated, and all solutions were evaporated in N_2 under reduced pressure at <35°.

Whole myrobalans, the dried fruit of *Terminalia chebula* Retz, grown in the Jubbulpore district of the Madhya Pradesh, were split open, the kernels were removed and the remaining tissue was pulverized (Hathway, 1956).

Preparation of cell-wall material

Pulverized myrobalans (100 g.) were extracted with several changes of fresh methanol in a Soxhlet apparatus until successive extracts were colourless. The residual material was then air-dried and ground to 60-mesh in a Wiley mill. The ground meal was then extracted with 89 % ethanol in a Soxhlet apparatus until sugars could not be detected in the last extract when treated with the anthrone reagent (Hathway, 1956). The final product was dried successively in a current of air, and over P_2O_5 at 20° *in vacuo* for 4 days. Yield, 18–19 g.

Cell-wall material prepared in this way contained polysaccharides (chiefly pectic material) originally present in the cell sap of the ripe fruit.

Fractionation of cell-wall material

The results of the analysis to which duplicate samples of cell-wall material were submitted are presented in Table 1. Water-soluble polysaccharides, equivalent to 'pectin', were removed with hot water, and, after chlorite delignification, the remaining 'holocellulose' was fractionated by

successive extraction with N- and 4N-KOH. The fractions of mixed polysaccharides obtained were all analysed for ash and, after hydrolysis, for their constituent monosaccharides and D-galacturonic acid.

A preliminary extraction of 10 g. of the cell-wall preparation with four successive volumes of 200 ml. of cold water removed most of the residual yellow pigment (600 mg.) and some pectin (270 mg.). The former appears as an un-numbered spot on the chromatogram shown as fig. 1 by Hathway (1956), where it has an R_F value of 0·22 in the first-way solvent system and remains adjacent to the ordinate in the second-way solvent system. The pectin was recovered free from this pigment by precipitation of the aqueous extract with 2·5 l. of ethanol.

The fraction which was insoluble in cold water was extracted with 750 ml. of water at 100° for 12 hr. The mixture was filtered through a sintered-glass funnel and the residue was washed successively with five 200 ml. portions of boiling water and a large volume of acetone. The yield of insoluble material was 6·58 g. The combined filtrate and washings were evaporated to 50 ml. and the polysaccharides were precipitated with ethanol. The yield of 'pectin' soluble in hot water was 2·64 g. 'Pectin' from the cold- and hot-water extracts was combined for the purpose of hydrolysis.

Extraction of the cell-wall material with aqueous solutions of ammonium oxalate or sodium polyphosphate (Allbright and Wilson Ltd., Oldbury, Worcs.) did not remove a greater quantity of polysaccharide.

The fractionation of the water-insoluble material, and the hydrolysis of all the cell-wall fractions, were carried out by the methods of Jermyn & Isherwood (1956), and the hydrolysates were freeze-dried.

Analysis

Sugars in the hydrolysates. These were resolved by single-way paper chromatography effected by the descending method. Longitudinal strips were cut from each paper and sprayed with a *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) or *p*-anisidine phosphate solution in water-saturated butan-1-ol in order to locate the sugars on the unsprayed papers, which were cut into transverse strips for the purpose of elution and estimation (Flood, Hirst & Jones, 1947, 1948; Hawthorne, 1947; Jermyn & Isherwood, 1949). In the present work, the following solvent systems were employed: propan-1-ol–ethyl acetate–water (7:1:2, by vol.) (Albon & Gross, 1952; Hathway, 1956); ethyl acetate–acetic acid–water (3:1:3, by vol.) (Jermyn & Isherwood, 1949); the organic phase of benzene–butan-1-ol–pyridine–water (1:5:3:3, by vol.). The third solvent system, which resolves galactose and glucose, was used in the exploratory stages of the work to confirm the absence of galactose from the hydrolysates. Individual monosaccharides and D-galacturonic acid were estimated by a modified ferricyanide (Hagedorn & Jensen, 1923) method, unpublished details of which were kindly made available to us through the courtesy of Dr A. E. Flood.

The polygalacturonic acid content of the combined 'pectin' fractions was also determined by the calcium pectate method (Nanji & Norman, 1928).

Compounds other than polysaccharides in the cell wall

These were determined in each fraction, and the figure for the carbohydrate content was amended accordingly. Some error is involved. Thus the possible presence of water-insoluble protein which is destroyed by chlorite oxidation (Wise, Murphy & D'Addieco, 1946) will enhance the apparent lignin figure, and a lower value was obtained by the hydrolysis method (Ritter, Seborg & Mitchell, 1932). The yellow pigment was estimated directly, and the possible presence of undetected impurities would lower this figure.

RESULTS

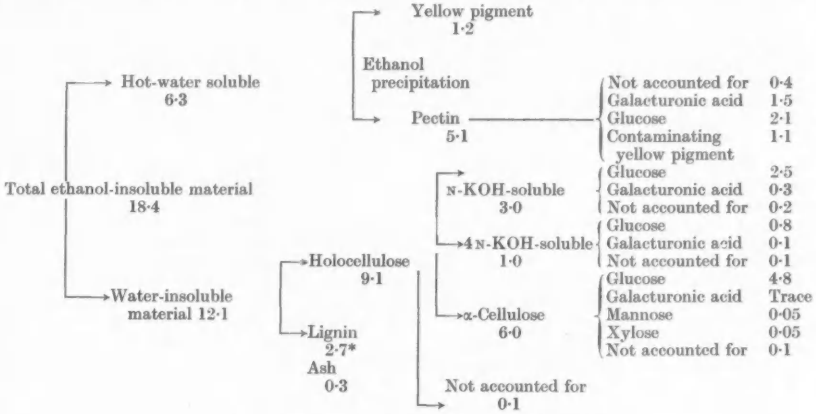
The cell-wall polysaccharides were fractionated by a series of analytical operations (Table 1) (Jermyn & Isherwood, 1956; O'Dwyer, 1923, 1926, 1928; Ritter & Kurth, 1933), which afforded fractions of mixed polysaccharides. These fractions were hydrolysed by the methods of Jermyn & Isherwood (1956) and the corresponding monosaccharides were separated by paper chromatography and estimated by established methods. For myrobalans it was readily established that the galacturonic acid can be accounted for as polygalacturonic acid, and the bulk of the glucose as cellulose. The remainder of the glucose is derived from starch, which is treated by the analytical procedure as a cell-wall constituent and which is consequently omitted from Table 2 which lists the cell-wall polysaccharides. The only other sugars present in the hydrolysates are traces of mannose and xylose, which correspond to the presence of small quantities of mannan and xylan in the cell wall. The polygalacturonic acid figure was checked by the calcium pectate method, and also by the isolation of the polyuronide. This procedure checked in the present context the overall assumption of Jermyn & Isherwood's (1956) methods. Hydrolysis of the isolated cellulose fraction showed that it was virtually pure polysaccharide. Cellulose-breakdown products occur in the potassium hydroxide-soluble fractions, and these figures together with that for cellulose appear as 'holocellulose' in Table 2.

DISCUSSION

The present investigation of myrobalans deals with a large cell-wall fraction compared with that of such fruit as the apple (Smith, 1935; Norman, 1937; Kidd & West, 1939; Kidd, West, Griffiths & Potter, 1940; Echevin, 1941), pear (Hansen, 1939; Jermyn & Isherwood, 1956) and tomato (Clendenning, 1941). Moreover, the high cellulose and lignin contents of the cell wall, and the enormous cellular content of tannin extractives, are consistent with the fact that the dried fruit cannot be cut with a knife. Myrobalans which are picked before or at the climacteric (Chopra, 1939; Edwards, Badhwar & Dey, 1952; Howes, 1953) have subsequently undergone autolysis, accelerated by storage under tropical conditions, and this is confirmed by the presence of 'pectins' soluble in cold and hot water.

Table 1. *Fractionation of cell-wall material from myrobalans*

Figures are calculated as percentage of initial weight of moisture-free fruit. Sugars are expressed in the anhydro form.



* 2·2 by the method with sulphuric acid (see text).

Table 2. *Total amount of each polysaccharide in the myrobalans cell-wall (minus the soluble glucosan)*

Polysaccharide	Amount of each polysaccharide in the moisture-free fruit (%)
Holocellulose glucosan* (cellulose)	7·85
Mannan	0·05
Polygalacturonic acid†	1·9
Xylan	0·05
Total	9·85

* Estimation of starch in the N-KOH-soluble fraction showed that the glucosan of this fraction was distributed between starch and holocellulose in the ratio of 1:9.
† 1·5 by the calcium pectate method (see text).

It was first noticed by Frémy (1840) that insoluble protopectin which had been laid down in the primary cell walls during the growth phase of fruit was converted by protopectinase into soluble pectin during senescence. Later, Barnell (1943) found a depletion in the hemicelluloses of ripening banana, and in their outstanding study Jermyn & Isherwood (1956) reported that the principal labile materials during the breakdown of the pear cell wall are araban and galactan, together with a significant amount of holocellulose glucosan. The absence of arabinose and the presence of only a very small proportion of xylose in the hydrolysates of the cell-wall fractions show that hemicellulose components are virtually absent from dried myrobalans. This also applies to galactan. Althcugh such hemicelluloses and galactan may have undergone rapid physiological change in the isolated tissue during drying, the absence of galactose and the presence of only trace quantities of arabinose and xylose in the polyol fraction of the extractives (Hathway, 1956) suggests that the hemicellulose content of the cell wall may have never been high. In agreement with the fact that a significant quantity of holocellulose glucosan becomes labile during senescence, a proportion of myrobalans holocellulose glucosan was found in the soluble fractions.

It is difficult away from the country of origin to attempt to follow the changes which occur in the cell-wall polysaccharides during the ripening of myrobalans. The analytical methods used in the present work are suitable for this project, which is particularly worthwhile since this crop or 'hirda' is one of the most highly esteemed of the world's tanning materials.

SUMMARY

1. The cell-wall fraction of myrobalans is large and holocellulose glucosan accounts for 8% of the dry weight of the fruit and polygalacturonic acid for a further 2%. Galactans and hemicelluloses are virtually absent.

2. The changes which occur after abscission in senescent myrobalans give rise to soluble pectin and cellulose-breakdown products which may be separated from the cell wall of the dried fruit.

The authors thank the Director and Council of the British Leather Manufacturers' Research Association for their permission to publish this work. We are also indebted

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The Influence of Tannins on the Degradation of Pectin by Pectinase Enzymes

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When a high proportion of the cell-wall polysaccharides of myrobalans was found to consist of polygalacturonic acid (Hathway & Seakins, 1958), it became important to establish whether the available pectinases effect the degradation of pectin and low-methoxy pectin in the presence of myrobalans gallo- and ellagi-tannins. The effect of gambir-tannin extractives which contain (+)-catechin and phlobatannin on the enzymic degradation of pectin and low-methoxy pectin was also investigated.

MATERIALS AND METHODS

Paper chromatography was effected in all-glass apparatus at a constant temperature of 25°. Chromatograms were dried at room temperature, unless otherwise stated.

Pectin (apple) 240-grade (British Drug Houses Ltd.) was purified by repeated extraction with aq. 70% (v/v) ethanol, and the residue was dried at 35° and contained 18·6% of OMe.

Low-methoxy pectin was prepared by treatment of 50 g. of the commercial pectin with a mixture of 188 ml. of aq. 95% (v/v) ethanol and 62 ml. of N-NaOH for 20 min., and the precipitate obtained was washed with a mixture of 100 ml. of 95% ethanol, 10 ml. of water and 5 ml. of N-HCl, and dried at 35°. Low-methoxy pectin contained <0·5% of OMe.

Pectin from the myrobalans cell-wall contained 16·0% of OMe.

Commercial pectinase preparations used in this work include Pectozyme (Norman Evans and Rais Ltd., Stockport, Ches.), Pectinol-100D (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), Pektinol klarlöslich doppelkonzentriert (Rohm und Haas A.-G., Darmstadt, Hessen, Germany). Since it was proposed to investigate the pectolytic action of pectic enzymes of fungal origin in the presence of hydrolysable tannins, it was necessary to determine whether they exhibit tannase activity, as tannase can be obtained from cultures of such fungi as *Aspergillus niger* which have been grown in the presence of myrobalans-tannin extractives (Freudenberg, 1921; Freudenberg & Vollbrecht, 1921). A 5% (w/v) soln. of myrobalans-tannin extractives was accordingly digested with a large excess of the three enzyme preparations at 25° for 1 week, when two-dimensional paper chromatograms of myrobalans extractives before and after enzyme treatment were compared (Hathway, 1956). The presence of such hydrolysable tannins as chebulagic and chebulinic acids and corilagin in approximately the same proportions on both sets of chromatograms suggests that these enzyme preparations are devoid of tannase activity. Known substrates of tannase include chebulinic acid and gallotannin.

Myrobalans-tannin extractives refer to the methanol extract of the ground meal and gambir-tannin extractives refer to the commercial extract derived from the harvested leaves of *Uncaria gambir* Roxb. Gallotannin was obtained free from *m*-digallic and gallic acid contamination by the continuous ethyl acetate extraction of a solution of tannic acid (British Drug Houses Ltd.) in aq. $NaHCO_3$ soln. in a Schacherl apparatus, followed by evaporation of the solvent (Paniker & Stiasny, 1911).

Action of pectinase on pectin. A 1% (w/v) soln. of the pectins was prepared. Since the salt concentration produced by the addition of adequate buffer to maintain a constant pH would prevent the resolution of the oligogalacturonic acids and D-galacturonic acid by paper chromatography, the pH was adjusted to 6·0 by the addition of small volumes of N-HCl or N-NaOH. During pectolysis, the pH of the pectin solutions was maintained by the addition of small volumes of N-NaOH to neutralize the additional free carboxyl groups which were liberated. Experiments with tannin extractives were conducted at pH 3·0, and such pectin solutions were centrifuged to remove traces of insoluble material. Pectin solutions (1%) were also employed for viscometry.

Measurement of viscosity. The progress of the early stages of pectolytic action was followed by measuring the change in rate of flow of pectin solutions through a no. 2 Ostwald-type viscometer, B.S. 188:1937 (J. W. Towers and Co. Ltd.). Enzyme digests and viscometric measurements were conducted at 25±0·05°. In carrying out experiments which emphasized differences in pectolytic activity, due to different enzymes or to the inactivation of enzyme by tannin, such factors as concentration and pH were kept constant in the comparable situations.

Chromatography of D-galacturonic acid and the oligogalacturonic acids

Samples (4 × 5 μl.) were withdrawn from digests during the course of pectolysis of low-methoxy pectin, and applied to appropriate positions on the starting line of a Whatman no. 1 filter paper, 57 cm. in length. This line of spots formed a time sequence from the beginning to the end of the reaction. Single-way chromatography was effected by the descending method, with water-saturated *iso*butyric acid (Jermyn & Tomkins, 1950) as solvent system. Papers were irrigated for 40 hr. or 60 hr., and dried (1 hr.) in an air current at 60–70°. Marker spots (4 × 5 μl.) of 0·4% (w/v) solutions of authentic di-, tri- and tetra-galacturonic acid and D-galacturonic acid respectively were used. Treatment with a $AgNO_3$–NaOH spraying reagent (Trevelyan, Procter & Harrison, 1950), followed by fixation in aq. 4% $Na_2S_2O_3$, was used to detect the acids, which gave intense black spots, as they possess 1:2-glycol groups (Hathway, 1956). Paper chromatography of those digests which contained tannin extractives, however, was effected with ethyl acetate–acetic acid–water (3:3:1, by vol.) mixture as solvent system. The papers were irrigated for 40 hr., and the ultimate product of reaction, D-galacturonic acid, was exposed as a red spot by means of the *p*-anisidine phosphate reagent. Duplicate experiments were set up for following the course of pectin hydrolysis. The first experiment was used for the detection of oligogalacturonic acids and D-galacturonic acid. In the second experiment, portions were adjusted to pH 9·0 and allowed to stand for 15 min., after which Na^+ ion was removed by Zeo-Karb 225 cation-exchange resin in the H^+ form, and a similar line of spots was made on a second chromatogram.

Gallotannin–pectinase complex. A soln. of Pektinol klarlöslich doppelkonzentriert (0·16 g.) in water (50 ml.) was treated with excess of a 20% solution of gallotannin and centrifuged. The sediment was emulsified with water and separated in the centrifuge. The change in rate of flow of a 1% pectin soln. of pH 6·0 was measured during pectolysis by the gallotannin–enzyme complex, the original enzyme preparation and the enzyme regenerated from another portion of the gallotannin–enzyme complex by treatment with aq. 80% (v/v) acetone.

RESULTS

The present work represents an investigation of the effect of tannins on the pectolytic action of pectinase enzymes. A qualitative exploration of the course of pectolysis by means of paper chromatography showed that with all three enzyme preparations tested, and with low-methoxy pectin, a series of up to ten spots appeared on the developed chromatogram (Fig. 1). The fastest-moving spot, D-galacturonic acid, appeared simultaneously with the slower-moving ones, and it increased steadily in size and intensity until the end of the reaction, when it was the sole surviving spot. A typical pectolysis was complete within 3 or 4 days. The slower-moving spots waxed in intensity and size, and then waned and disappeared in the course of hydrolysis. Pectolysis of the selected substrate followed the same pattern in all three cases, and these enzyme preparations contrasted with Brooks & Reid's (1955) (D)-type enzyme and Schubert's (1952*a*, *b*) α-polygalacturonase, which gave a mixture of di- and tri-galacturonic acid and D-galacturonic acid. Parallel gelling tests with ethanol and calcium salts, however, showed that the colloidal properties disappeared more rapidly with Pectinol-100D than with Pektinol klarlöslich doppelkonzentriert. The more crude Pectozyme preparation was used only in the exploratory stages of the work.

Ashby, Brooks & Reid (1955) have recently isolated di-, tri- and tetra-galacturonic acid from enzyme hydrolysates, and the size and structure of these oligogalacturonic acids have been established (Jones & Reid, 1954, 1955). Di-, tri- and tetra-galacturonic acid have been used as markers in the present work, and they correspond to the second, third and fourth fastest-moving spots respectively. The regularity of the successive R_F values of galacturonic acid, di-, tri- and tetra-galacturonic acid, and of the remaining oligogalacturonic acids, suggests a series of increasing molecular complexity. When the R_M values [log $(1/R_F - 1)$] of this series were calculated (Bate-Smith & Westall, 1950) and plotted against the size of polymer a linear relationship resulted

(Fig. 2). Hence there is a strong supposition that the spots exposed by development of the chromatograms may be identified as a series of oligogalacturonic acids up to the decamer.

A similar investigation of the pectolytic action of these enzymes on substrates of apple and myrobalans pectin, however, showed that galacturonic acid gradually accumulated during the reaction, and no oligogalacturonic acids were detected. Further, when portions removed from these enzyme digests at intervals were treated with alkali before paper chromatography of the cation-free solutions, the developed chromatograms again showed an absence of polyuronide intermediates, and a gradual accumulation of galacturonic acid. The course of pectolysis of these pectins therefore differs profoundly from that of low-methoxy pectin.

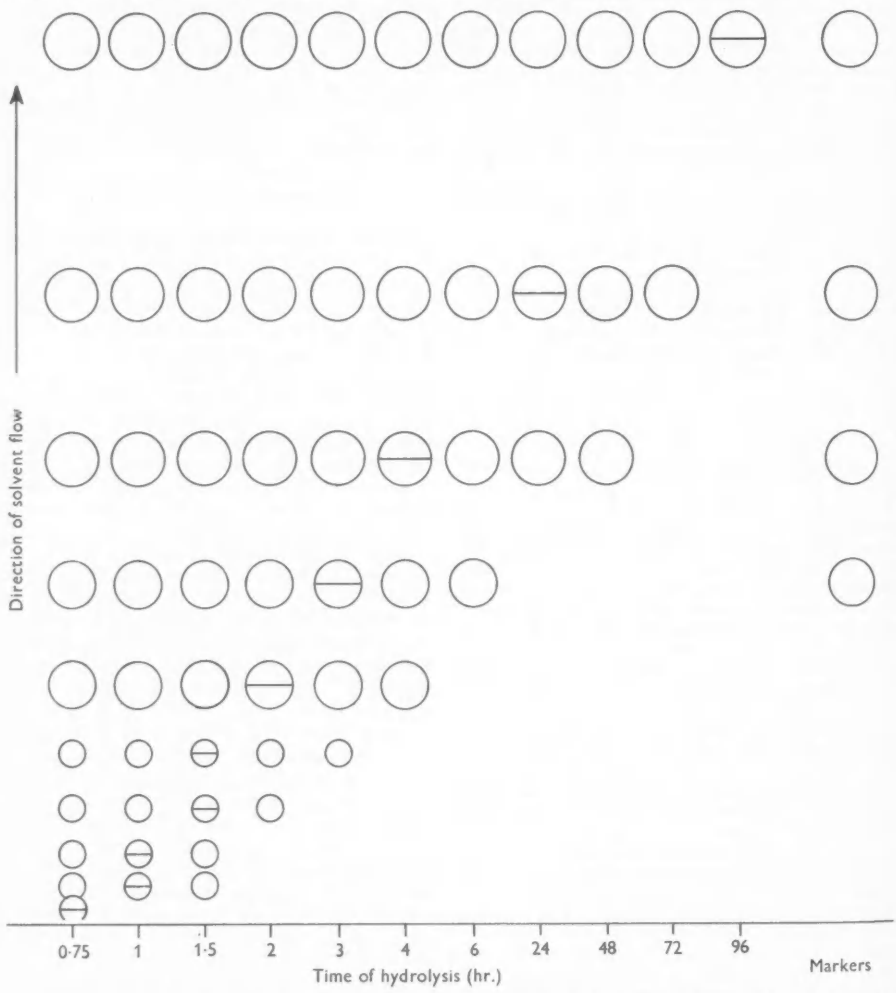

Fig. 1. Sequence of low-galacturonic acid polymers and D-galacturonic acid obtained when a 1% solution of low-methoxy pectin, pH 6·0, is acted upon by Pectinol-100D. This figure has been prepared from a chromatogram run at 25° for 40 hr. on Whatman no. 1 paper, in water-saturated *iso*butyric acid. For details see Methods. ⊖ Represents the spot of maximum intensity.

The progress of the initial stages of hydrolysis may be followed by measuring the change in time of flow of pectin solutions through an Ostwald-type viscometer tube. Such measurement, which is a function of viscosity, is dependent on the amount of unchanged pectin present. A comparison of the pectolytic action of Pectinol-100D and Pektinol klarlöslich doppelkonzentriert by these means is given in Fig. 3, for apple-pectin substrate at pH 6·0. The viscosity of the pectin solution falls rapidly until it approaches the value for water, when approximately 10% scission has occurred.

The presence of myrobalans tannins exerts an immediate inactivating influence on the pectolytic action of these enzyme preparations when a low-methoxy pectin solution of pH 3·0 is used as substrate (Fig. 4). When apple or myrobalans pectin is employed as substrate, a marked increase in viscosity is associated with the interaction between pectin and tannin, and in the course of hydrolysis the presence of myrobalans tannins exerts only a slight retarding influence on the pectolytic action. The rate of flow of the pectin solution falls steeply (Fig. 4). With pectin and low-methoxy pectin, pectolysis does, however, go to completion in the presence of myrobalans tannins. The presence of

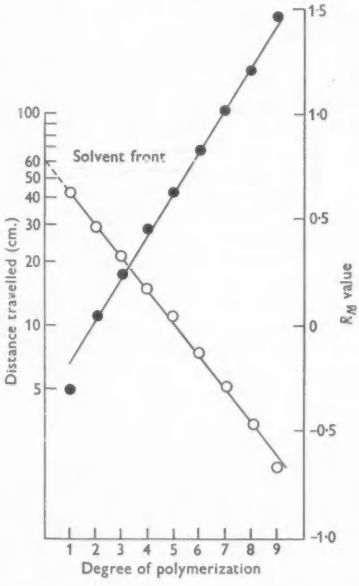

Fig. 2. Change in R_M value with size of galacturonic acid polymer. Degree of polymerization is plotted against the distance travelled by the corresponding spot (○), and solvent front, and against the R_M value (●).

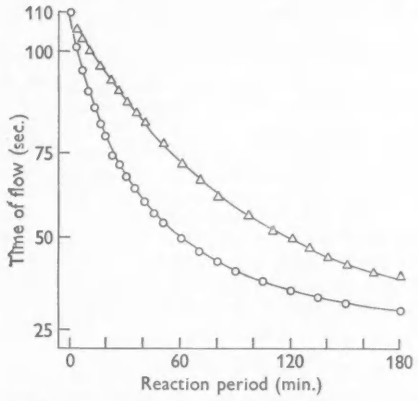

Fig. 3. Change in flow-rate of pectin solutions, produced by pectolytic action. The reaction flask contained a solution of 1% apple pectin, pH 6·0. ○, With 0·04% Pectinol-100D; △, with 0·16% Pektinol klarlöslich doppelkonzentriert. The ordinate refers to the time of flow through an Ostwald-type viscometer (for details see Methods).

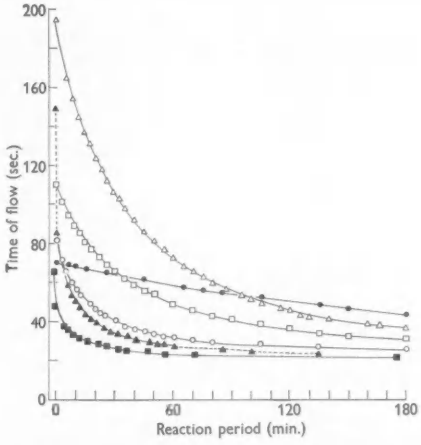

Fig. 4. Effect of myrobalans and gambir tannins on the change in flow-rate of pectin solutions brought about by pectolytic action. The reaction flask contained a solution of 1% low-methoxy pectin and 0·04% of Pectinol-100D, pH 3.0. ○, Without tannin; ●, with 5% myrobalans-tannin extractives; ■, with 5% gambir-tannin extractives. A solution of 1% pectin and 0·04% of enzyme was used under similar conditions. □, Absence of tannin; △, presence of 5% myrobalans-tannin extractives; ▲, 5% gambir-tannin extractives. The ordinate refers to the time of flow through an Ostwald-type viscometer (for details see Methods).

gambir tannin has no effect on the pectolysis of pectin and low-methoxy pectin.

When a solution of Pektinol klarlöslich doppelkonzentriert was precipitated by gallotannin, the resulting enzyme–tannin complex exhibited no initial pectolytic activity, but as the complex began to dissociate in a pectin solution, hydrolysis commenced (Fig. 5). The enzyme, regenerated from the enzyme–tannin complex by solvent precipitation, possessed a reasonable proportion of its original pectolytic activity. This experiment attempts to simulate the extreme case in which the presence of a big preponderance of gallotannin might be expected completely to impede pectolysis.

DISCUSSION

The evidence suggests that the spots, encountered in the chromatographic study of the pectolysis of low-methoxy pectin by the commercial enzyme preparations, may be identified as a series of oligogalacturonic acids up to the decamer. In this reaction, the action of two enzymes, endopolygalacturonase (endo-PG) and exopolygalacturonase (exo-PG) (Demain & Phaff, 1957) can be distinguished. Endo-PG attacks low-methoxy pectin in preference to pectin by a random mechanism of hydrolysis, which leads to the series of intermediate oligogalacturonic acids, and eventually to digalacturonic acid; exo-PG attacks low-methoxy pectin in preference to pectin by a terminal mechanism of hydrolysis. Thus in the present reaction, the action of exo-PG accounts for the appearance of galacturonic acid simultaneously with the oligogalacturonic acids produced by endo-PG. Further, the accumulation of a large amount of galacturonic acid before the formation of the intermediate oligogalacturonic acids when the enzyme level is lowered is readily understood on the assumption that two enzymes are involved in this reaction. Exo-PG also functions in the terminal removal of galacturonic acid units from the intermediate oligogalacturonic acids, and in the final scission of digalacturonic acid. The viscometric measurements showed that gambir tannin has no effect on the pectolytic action of endo-PG and exo-PG, whereas the immediate inactivation of pectolytic action in the presence of myrobalans tannins suggests that they inhibit the action of endo-PG.

The absence of oligogalacturonic acids and their methyl esters from the hydrolytic products of pectin can only be interpreted by the action of exopolymethylgalacturonase (exo-PMG) which attacks pectin in preference to low-methoxy pectin by a terminal mechanism of hydrolysis (Demain & Phaff, 1957). Some exo-PG action may also be involved in the overall hydrolysis, depending on the degree of methylation of the pectin. The hydrolytic action of exo-PMG is not affected by the presence of gambir tannin, but is slightly inactivated by myrobalans tannins.

These results show that the commercial enzyme preparations, Pectinol-100D and Pektinol klarlöslich doppelkonzentriert, are mixtures of at least three enzymes, endo-PG, exo-PG and exo-PMG. The results imply that the enzyme preparations would function efficiently in such natural substrates as blackcurrant juice, which contains phenolics and phlobatannin similar to those found in the gambir extractives. The fact that pectolysis can take place in the presence of myrobalans tannins is consistent with the ripening of the fruit, and the retarding action exerted by these tannins on the action of the pectolytic enzymes explains why sound, dry myrobalans can often be collected from the forest floor.

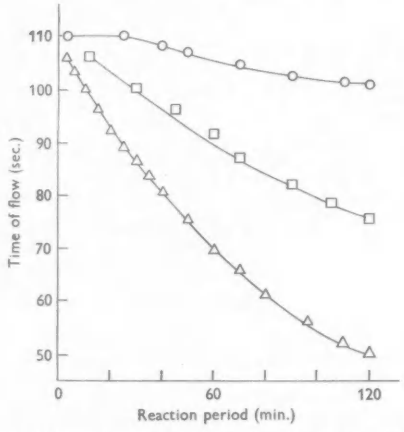

Fig. 5. Change in flow-rate of a 1% apple-pectin solution, pH 6·0, produced by the pectolytic action of a gallotannin–enzyme complex. ○, Complex derived from a 0·16% solution of Pektinol klarlöslich doppelkonzentriert; □, the enzyme regenerated from the complex (for details see Methods); △, the similar pectolytic action of the original enzyme.

SUMMARY

1. Pectolysis of low-methoxy pectin by commercial enzyme preparations leads to a series of oligogalacturonic acid intermediates up to the decamer, and eventually to D-galacturonic acid, whereas the enzymic hydrolysis of pectin affords D-galacturonic acid alone.

2. The encountered pectolytic actions of these enzyme preparations may be explained in terms of endopolygalacturonase (endo-PG), exopolygalacturonase (exo-PG) and exopolymethylgalacturon-

ase (exo-PMG). Endo-PG attacks low-methoxy pectin by a random mechanism of hydrolysis which leads to intermediate oligogalacturonic acids and eventually to digalacturonic acid, whereas exo-PG removes terminal galacturonic units from the substrate and its intermediate polyuronides, and cleaves digalacturonic acid. Exo-PMG attacks pectin by a terminal mechanism of hydrolysis.

3. Viscometric measurement shows that myrobalans tannins exert a powerful inactivation on the early stages of the pectolysis of low-methoxy pectin, but only a slight retarding action on the pectolysis of pectin.

4. The fact that these enzymic hydrolyses go to completion in the presence of myrobalans tannins is consistent with the ripening of the fruit.

5. Gambir tannin has no effect on the pectolytic actions investigated.

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Distribution of Ascorbic Acid in Normal and Leukaemic Human Blood

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It has been known for some time that the buffy layer of normal human blood contains a high concentration of ascorbic acid (Butler & Cushman, 1940, 1941). The distribution between lymphocytes, granulocytes and platelets has, however, remained obscure and forms the subject of this present paper. Whereas previous workers have quoted their results on a wet-weight basis only, the data presented here are based on the analysis of individual cells determined by direct counting.

Leukaemic blood has been found to contain low concentrations of ascorbic acid, both in the serum (Hagtvet, 1945) and plasma and in the buffy layer (Waldo & Zipf, 1955). In contrast, Butler & Cushman (1940) found higher amounts than normal in the buffy layer. We have included analyses of chronic leukaemic human blood in this work, in order to investigate these claims and also to provide data on the relatively pure suspensions of lymphocytes and granulocytes obtainable in leukaemic diseases.

METHODS

Collection of blood. Blood (30–90 ml.) was aspirated from an antecubital vein with sterile paraffined all-glass syringes and no. 18 B.D. steel needles. It was immediately transferred to siliconized glass centrifuge tubes (10 ml.) containing as anticoagulant one part of disodium ethylenediaminetetra-acetate dihydrate (Sequestrene) soln. [1% (w/v) in 0·7% NaCl soln.] to nine parts of blood. The tubes were inverted several times to mix the contents.

Centrifuging. A horizontal refrigerated centrifuge, radius 14 cm., at a temperature of 4°, was used throughout.

Separation of the formed elements

Platelets. The whole blood was centrifuged at 800–1000 rev./min. for 10–15 min. The upper three-quarters of the supernatant platelet-rich plasma was carefully removed with siliconized Pasteur pipettes and transferred to polythene centrifuge tubes. (The blood remaining after removal of the platelet-rich plasma is called 'platelet-poor blood'.) After removal of a small measured quantity (about 0·01 ml.) for a direct platelet count (Barkhan, 1957), the measured volume of plasma was centrifuged at

3000 rev./min. for 15 min. to obtain a platelet 'button', which, after the removal of the supernatant plasma, was weighed. A platelet count on the platelet-poor plasma was performed as described above. The total number of platelets in the button was calculated from the difference between the platelet-rich and platelet-poor plasma counts and the total volume of the plasma. Microscopic examination of Leishman-stained films of the platelet-rich plasma showed a very occasional erythrocyte and the complete absence of leucocytes. The absolute number of erythrocytes was determined at the same time as the platelet count. The ratio erythrocytes:platelets was generally of the order of 1–3:1000; this small number would not contribute significantly to the weight of the platelet button.

Erythrocytes. About 10 ml. of platelet-poor blood was centrifuged in a Nylon centrifuge tube (1·5 cm. diam. × 10 cm.) for 30 min. at 3000 rev./min. The erythrocyte mass was obtained by piercing the base of the tube with a syringe needle and drawing off the erythrocyte layer, the top 1 cm. containing the leucocytes and remaining platelets being discarded. The number of erythrocytes was determined by direct counting. Examination of Leishman-stained films showed a concentration of less than 0·1% of leucocytes.

Leucocytes. The remaining platelet-poor blood was further centrifuged for 15 min. at 3000 rev./min. in several tubes (1·5 cm. × 10 cm.). After removal of three-quarters of the supernatant, the remaining plasma together with the top layers of cells containing the buffy layer was transferred with a Pasteur pipette to a narrow glass tube (15 cm. × 0·8 cm.) which was centrifuged at 3000 rev./min. for 1–2 hr. This produced an elongated buffy layer which could be separated from the underlying red-cell mass. The layer consisted of platelets and leucocytes but still contained a relatively large number of erythrocytes. This buffy-layer preparation was further suspended in a known volume of platelet-poor plasma (2–6 ml.) and the absolute number of each of these elements in the suspension was determined by direct counting, the platelets and erythrocytes as described above and the leucocytes by the standard method (Whitby & Britton, 1957). The measured volume of suspension was further centrifuged at 3000 rev./min. for 20 min. The packed cells were weighed after completely decanting the supernatant cell-free plasma. With normal blood the leucocytes consisted of both the poly- and mono-nuclear types.

Plasma. The platelet-poor plasma obtained after separation of the platelet button was used for the ascorbic acid estimation. This plasma contained some platelets (generally less than 10 000/mm.^{3}) but no leucocytes or erythrocytes.

Cell counts on whole blood

Leucocyte and platelet counts were made on each specimen of whole blood by the methods already described. An haematocrit value was also obtained (Wintrobe, 1956).

Ascorbic acid analyses

The platelet button and the buffy layer were extracted by stirring for a few minutes with metaphosphosphoric acid solution [3 ml. of 5% (w/v) solution/50 mg. of cells], and spinning till clear and removing the supernatant. The plasma and erythrocyte mass were mixed and allowed to stand with metaphosphoric acid solution [12 ml. of 5% (w/v) solution/4 ml. of cells or plasma] for a few minutes and then filtered. Portions (4 ml.) were used for ascorbic acid determinations by the 2:4-dinitrophenylhydrazine method of Roe & Kuether (1943) as modified by Bolin & Brook (1947). Most analyses were performed on the same day that the blood was taken. A few specimens were stored at −20° for up to 3 days. Some additional estimations on the platelet extract were made by the 2:6-dichloroindophenol method of Mindlin & Butler (1937).

Calculation of results

Plasma, erythrocyte and platelet values were calculated on the basis of their being pure preparations, since other components would not have contributed significantly to the amount of ascorbic acid estimated. For leucocytes, the value taken was that remaining after subtracting from the buffy-layer value the amount contributed by erythrocytes and platelets. Since the number of cells in each sample extracted had been determined it was possible to calculate the amount of ascorbic acid present in 10^{12} erythrocytes, leucocytes and platelets. Concentrations of ascorbic acid in units of mg./100 g. were calculated from the quantity of ascorbic acid in 10^{12} cells and then their weight. The weight of leucocytes was taken to be 894 g./10^{12} cells and was calculated from the known volume of leucocytes (1046μ^3 for granulocytes and 594μ^3 for lymphocytes; Blitzen & van den Berghe, 1945), assuming their specific gravity to be unity and the proportion of granulocytes to lymphocytes to be 2:1 (Wintrobe, 1956). For leukaemic leucocytes, the concentrations (mg./100 g.) were those obtained experimentally, since the buffy layers consisted almost entirely of leucocytes. The weight of platelets was taken to be 16 g./10^{12} platelets and was calculated from a mean platelet volume of 16 ml./10^{12} platelets determined experimentally and assuming a specific gravity of unity. The contribution of plasma and erythrocytes to the ascorbic acid concentration of whole blood was calculated from the haematocrit value and the ascorbic acid concentration in each constituent. The contributions of the leucocytes and platelets were calculated from the whole-blood cell count and the ascorbic acid in each cell.

Mean platelet volume

The mean platelet volume of six normal subjects was determined with a microhaematocrit adapted to hold a large volume of liquid. Platelet-rich plasma (2 ml.) containing a known number of platelets was spun at 3000 rev./min. until the volume of packed cells was constant (3–4 hr.). The length of the column of platelets was read off on the scale and the volume obtained by calibrating the microhaematocrit with water. The mean platelet volume was 16·0±0·7μ^3 and the range 13·3–18·1μ^3. The absolute value may have been lower than the results obtained owing to the difficulty of securing maximum platelet packing. Tocantins (1938) quotes 10–12μ^3; Franke & Horwitz (1938) found 12·5μ^3 for pregnant women. The value of 7·3μ^3 obtained by Olef (1937) is likely to be too low as the platelet counts were about twice as high as those considered to be normal.

Saturation of subjects with ascorbic acid

Normal subjects were dosed orally in the evening with 1 g. of ascorbic acid in water for 4 consecutive days and the blood was taken in the morning. Ascorbic acid analyses of urine by the method of Bessey & King (1933) indicated

that the subjects were saturated. Saturation tests on several leukaemic subjects were conducted by the method of Abassy, Harris, Ray & Marrack (1935).

RESULTS

Normal subjects: before and after saturation with ascorbic acid

Concentrations of ascorbic acid found in the constituents of human blood from subjects before and after saturation with ascorbic acid are shown in Table 1. The concentrations in the platelets and leucocytes were high and they contained approximately 20 times as much as the plasma or erythrocytes. In the platelets, ascorbic acid (in mg./100 g.) had almost the same concentration as in the leucocytes. For platelets, additional analyses were carried out by the 2:6-dichlorophenol method and the results agreed quite closely with those given by the 2:4-dinitrophenylhydrazine method.

On saturation of these subjects with ascorbic acid, the concentration was almost doubled in the plasma, erythrocytes and leucocytes and increased by 30% in the platelets.

Table 1 shows the ascorbic acid content of the cellular constituents expressed as mg./10^{12} cells. Leucocytes, because of their larger size, had by far the largest concentration per cell. Although the platelet is approximately one-tenth the volume of the erythrocyte there was about six times more ascorbic acid in each platelet.

The contribution of each constituent to the ascorbic acid content of whole blood is shown in Table 2. The results are expressed both as mg./100 ml. of blood and as percentages of the total amount. No determinations were made on whole blood, but the total ascorbic acid was calculated from the individual constituents.

The contribution of the plasma and erythrocytes accounted for about 80% of the total ascorbic acid in blood. This compared with 10% for the platelets and 10% for the leucocytes. On saturation with ascorbic acid, the increase was slightly more in the plasma than the other blood constituents.

Leukaemic blood

Determinations were made on the blood from three patients with chronic myeloid leukaemia and two with chronic lymphatic leukaemia (Table 3). The leucocyte counts of these patients were high (50 000–500 000/mm.^{3}), and the cells consisted of at least 95% of the granulocytic or lymphatic types respectively. One of the chronic myeloid leukaemia patients and the two chronic lymphatic leukaemia patients had no measurable ascorbic acid in the plasma ($<0{\cdot}02$ mg./100 ml.), and only very small amounts or none in the erythrocytes; the other two cases of myeloid leukaemia had fairly low values in the plasma and erythrocytes. The platelets and leucocytes of all patients, however, contained an appreciable amount of ascorbic acid. Because of these findings, one of the myeloid and the two lymphatic leukaemic patients were given an ascorbic acid-loading test. It was found that a longer time (6–14 days) was needed to attain saturation than in normal subjects, who usually reach saturation in 2 days (Abassy *et al.* 1935). After saturation, the ascorbic acid contents of the constituents of the blood were within the normal range for saturated subjects (Table 3). This applied particularly to the leucocytes when the concentration was expressed in mg./10^{12} cells. Chronic myeloid and lymphatic leukaemic leucocytes contained about the same amount of ascorbic acid per cell. Since the volume of the granulocytes is nearly twice that of the lymphocytes (Tivey, Li & Osgood, 1951), the concentration expressed as mg./100 g. is larger in the lymphocytes than in the granulocytes.

DISCUSSION

From the above results it is clear that platelets and leucocytes contribute almost equally to the high concentration of ascorbic acid in the buffy layer of human blood. Whilst it is generally believed that leucocytes have a high content, the possibility that platelets are also rich in ascorbic acid has received little attention. We have found that the concentration in the platelets is about the same as that in the leucocytes.

From a functional aspect, ascorbic acid may play an important role in the activity of leucocytes. Thus in scorbutic guinea pigs the phagocytic activity of leucocytes is said to be lowered (Lawrynowicz, 1931; Cottingham & Mills, 1943). Nungester & Ames (1948) have also claimed that the phagocytic activity of leucocytes of peritoneal exudates bears a direct relationship to their ascorbic acid content. For platelets, no evidence has so far been obtained that ascorbic acid influences their activity. Thus in scorbutic guinea pigs the activity of platelets in thromboplastin formation and in syneresis is normal (P. Barkhan & A. N. Howard, to be published). One of the functions of platelets may be as a carrier of pharmacologically active materials (Humphrey & Jaques, 1954). This concept has been used to explain the large quantities of adrenaline (Weil-Malherbe & Bone, 1954), serotonin (Bigelow, 1954) and histamine (Humphrey & Jaques, 1954) which they contain. Platelets could also be considered as a means of vitamin C transport to sites of injury.

There is increasing support for the concept that intravascular blood coagulation is a continuous

Table 1. *Ascorbic acid concentration in the constituents of human blood of normal and saturated subjects*

Mean values with the standard errors of the means and the ranges are given. Ascorbic acid results were obtained by the 2:4-dinitrophenylhydrazine method, with the exception of results in column *B*, where the 2:6-dichloroindophenol method was used.

		Ascorbic acid							
		(mg./100 ml.)		(mg./100 g.)			(mg./10^{12} cells)		
				Platelets					
Subjects	No.	Plasma	Erythrocytes	*A*	*B*	Leucocytes	Erythrocytes	Platelets	Leucocytes
Normal	6	0·69±0·06 (0·50–0·89)	0·69±0·05 (0·53–0·83)	18·3±1·4 (13·2–22·8)	18·6±1·2 (14·8–22·8)	16·5±2·14 (12·1–25·1)	0·55±0·04 (0·42–0·66)	2·91±0·22 (2·11–3·65)	148±19 (108–224)
Saturated with ascorbic acid	6	1·24±0·11 (0·85–1·62)	1·15±0·07 (0·92–1·41)	24·3±1·8 (20·6–32·8)	—	27·7±1·9 (20·3–32·5)	0·92±0·06 (0·73–32·5)	3·90±0·29 (3·32–5·24)	248±17 (182–290)

Table 2. *Contribution of each constituent to the ascorbic acid concentration of whole blood*

Mean values per 100 ml. of blood and per cent. of total ascorbic acid, with the standard error of the mean, are given. Ascorbic acid was estimated by the 2:4-dinitrophenylhydrazine method.

		Ascorbic acid								
		Plasma		Erythrocytes		Platelets		Leucocytes		Total
Subjects	No.	(mg./100 ml.)	(%)	(mg./100 ml.)	(%)	(mg./100 ml.)	(%)	(mg./100 ml.)	(%)	(mg./100 ml.)
Normal	6	0·34±0·04	40·2±3·2	0·35±0·04	40·7±3·0	0·08±0·003	9·5±0·8	0·08±0·009	9·7±1·1	0·84±0·05
Saturated with ascorbic acid	6	0·68±0·06	45·9±2·1	0·52±0·03	36·4±3·7	0·11±0·02	7·7±1·0	0·15±0·01	10·1±1·3	1·46±0·08

Table 3. *Ascorbic acid in the blood of leukaemic subjects*

				Ascorbic acid						
				(mg./100 ml.)		(mg./100 g.)		(mg./10^{12} cells)		
Subject	Type of chronic leukaemia	Ascorbic acid status	Time to saturate (days)	Plasma	Erythrocytes	Platelets	Leucocytes	Erythrocytes	Platelets	Leucocytes
Ba	Myeloid	Unsaturated	—	0·00	0·00	13·8	11·5*	0·00	2·2	101
Te	Myeloid	Unsaturated	—	0·56	0·20	15·6	23·9	0·16	2·5	162
Wa	Myeloid	Unsaturated	—	0·37	0·50	20·6	8·5	0·39	3·3	87
Wa	—	Saturated	8	1·04	1·36	24·4	22·1	0·83	3·9	298
Gi	Lymphatic	Unsaturated	—	0·10	0·36	6·9	21·7	0·29	1·1	62
Gi	—	After 10 days on saturation dose	—	0·00	—	16·9	38·3	—	2·7	190
Gi	—	Saturated	15	0·90	1·02	26·2	67·6†	0·82	4·2	274
Wd	Lymphatic	Unsaturated	—	0·00	0·16	19·2	61·6	0·13	3·1	265
Wd	—	Saturated	6	1·26	1·11	24·4	67·1	0·88	3·9	289

* 11·4 mg./100 g. by method of Mindlin & Butler (1937).

† 68·8 mg./100 g. by method of Mindlin & Butler (1937).

process under physiological conditions (Roos, 1957). This would explain the normal maintenance of haemostasis, the continuous utilization of the various clotting factors and the tendency to haemorrhage when the coagulation mechanism becomes defective either through deficiency of one or more clotting components or the presence of inhibitory substances. The platelet is an important component of the haemostatic mechanism. How it reacts with the vascular wall to prevent haemorrhage is not known; possibly it helps to form the fibrin film on the endothelium. If this involves deposition of platelets on the vascular wall, then this would constitute a means of carrying ascorbic acid to the site where it is believed to play an important part in the formation of the ground substance of the vascular membranes (Wolbach & Howe, 1926) or in the tissue sheath (Penney & Balfour, 1949), which are also important in maintaining vascular integrity.

Another pointer to the function of vitamin C in platelets may be seen in the similarity between the petechial haemorrhages observed in scurvy and those seen in thrombocytopenic purpura. The abnormality in scurvy, however, is not due to a quantitative deficiency of platelets since the platelet count is actually increased (Hess, 1920; P. Barkhan & A. N. Howard, to be published). These vascular defects may have a common origin in the failure of sufficient vitamin C to reach the vascular and capillary walls.

The ascorbic acid content of erythrocytes and plasma is low and the results are in agreement with those of other authors (Butler & Cushman, 1940). Owing to the much larger mass of erythrocytes and plasma compared with the buffy layer, they contribute up to 80% of the total in the blood.

High values for an indophenol-reducing substance have been reported in leukaemic blood (Stephen & Hawley, 1936; Cuttle, 1938), and the ascorbic acid concentration of leukaemic leucocytes is apparently higher than normal (Butler & Cushman, 1940). Our observations show that the ascorbic acid concentration of leucocytes is higher than normal in chronic lymphatic leukaemia but not in chronic myeloid leukaemia. Owing to the smaller volume of lymphocytes compared with granulocytes, the ascorbic acid content per cell is the same in both types of leukaemia. Data presented by Butler & Cushman (1940) are in accord with our results; they also found very high ascorbic acid concentrations in chronic lymphatic leukaemia. Whether or not this difference in ascorbic acid concentration exists between normal lymphocytes and granulocytes is as yet unknown. Although Waldo & Zipf (1955) found that the buffy layer in both acute and chronic leukaemia is low in ascorbic acid, their apparently conflicting results could be explained by the differences between the ascorbic acid nutrition of their subjects and ours.

We have confirmed the report of Waldo & Zipf (1955) that the ascorbic acid content of leukaemic plasma is low. In three of our cases no measurable ascorbic acid was found in the plasma, although the leucocytes and platelets did contain considerable amounts. Plasma ascorbic acid is considered a poor index of ascorbic acid nutrition (Sebrell & Harris, 1954) and for this reason three patients were submitted to the saturation test of Abassy *et al.* (1935). These subjects took a much longer period to saturate than is normal. Cuttle (1938) obtained similar results with two cases of acute and two of chronic myeloid leukaemia.

SUMMARY

1. The concentration of ascorbic acid in the plasma, erythrocytes, platelets and leucocytes of human blood has been determined in normal subjects, before and after saturation with ascorbic acid.

2. Both the platelets and leucocytes were found to have high concentrations (before saturation 15 mg./100 g.; after saturation 25 mg./100 g.) but the plasma and erythrocytes were comparatively low (before saturation 0·7 mg./100 ml.; after saturation 1·2 mg./100 ml.).

3. Because of the large mass of erythrocytes and plasma, they contributed about 80% of the total ascorbic acid in the blood. About 10% was contributed by the platelets and 10% by the leucocytes.

4. The concentration of ascorbic acid was higher in the chronic leukaemic lymphocyte (60 mg./100 g.) than in either the normal leucocyte (20 mg./100 g.) or leukaemic granulocyte (20 mg./100 g.); the total quantity in each cell was, however, the same in all these cell types.

5. It was confirmed that in chronic lymphatic (two cases) and myeloid (three cases) leukaemia the ascorbic acid concentration in the plasma is lower than normal.

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Thetin–Homocysteine Transmethylase

SOME FURTHER CHARACTERISTICS OF THE ENZYME FROM RAT LIVER

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In previous communications (Maw, 1955, 1956) it was shown that as the enzymic methylation of homocysteine by dimethylthetin, $(CH_3)_2\overset{+}{S}\cdot CH_2\cdot CO_2^-$, at pH 7·4 involves the appearance of one proton for each molecule of methionine formed, the reaction can therefore be followed manometrically in a bicarbonate medium by measuring the carbon dioxide evolved. The enzyme responsible has been named thetin–homocysteine transmethylase, and has been partially purified from rat liver. The effect on the activity of the rat-liver transmethylase of varying the concentrations of the two substrates at pH 7·4 has been studied.

The present paper deals with some further characteristics of the enzyme. A brief survey of its substrate specificity has been made, and the effects on its activity of metal ions and a number of the more common enzyme inhibitors have been examined. Homocysteine is specifically required as a methyl acceptor. This is in contrast with the less specific requirement for a methyl donor, for in addition to dimethylthetin chloride nine other sulphonium salts have so far been shown to have some activity as methyl donors.

Some properties of the thetin–homocysteine transmethylase from pigeon liver, including its inhibition by mercaptoacetate (thioglycollate), have been briefly reported by Sloane, Boggiano, Coulomb & Hutchings (1956), and Durell & Anderson (1956) have described the characteristics of the enzyme from horse liver. In these studies enzymic activity was followed by colorimetric determination of the methionine formed. During the completion of the experiments reported here, Durell, Anderson & Cantoni (1957) published details of the purification of the horse-liver transmethylase, in which methionine determinations were used as a measure of enzymic activity. Many of the characteristics of the enzyme described by these workers are in substantial agreement with those given below for the rat-liver enzyme.

MATERIALS AND METHODS

Substrates. The various thetins under study were prepared in the form of their chlorides or bromides by interaction of the corresponding chloro- or bromo-fatty acids with the appropriate thio ether (see Maw, 1953). Sulphocholine iodide (2-hydroxyethyldimethylsulphonium iodide) was prepared according to the method of Maw & du Vigneaud (1948c). The methyl ester of dimethylthetin iodide was obtained by the interaction of methyl methylthioacetate with methyl iodide. The methionine methylsulphonium chloride used was a gift from Professor F. Challenger, to whom the author wishes to express his thanks.

DL-Homocysteine was prepared by the reduction of DL-homocystine with sodium in liquid ammonia (Riegel & du Vigneaud, 1935–36).

Manometric procedure. The preparation of active fractions of thetin–homocysteine transmethylase from rat liver has been described in a previous communication (Maw, 1956). An account of the manometric method used to study the transmethylase has also been given in this earlier paper.

Solutions of substrates and other substances to be added to the enzyme preparation were prepared in 0·02 M-sodium bicarbonate solution. The thetins and other acidic compounds were first neutralized with dilute aqueous sodium hydroxide and the volume then made up with 0·04 M-sodium bicarbonate solution and water so as to give a final bicarbonate concentration of 0·02 M. The volume of enzyme preparation added to the side arm of each flask was 0·5 ml., unless otherwise stated, and the final volume of fluid in each flask was 2·5 ml.

Enzyme activity has been expressed in terms of the initial reaction rate, this being the volume of carbon dioxide liberated during the 10 min. period commencing 5 min. after the enzyme has been mixed with the substrates.

RESULTS

Substrate specificity

Specificity of the enzyme towards methyl donors. In addition to dimethylthetin chloride, a number of other methylsulphonium salts have been examined for their ability to act as methyl donors to homocysteine. Their effectiveness as substrates for the transmethylase has been assessed in terms of the initial rate of evolution of carbon dioxide obtained in a 0·02 M-sodium bicarbonate buffer at pH 7·4, with 0·03 M-DL-homocysteine and 0·1 M-sulphonium halide (unless otherwise stated). The results obtained with these and a number of related ethylsulphonium compounds are given in Table 1, and have been expressed as percentages of the rate obtained with 0·1 M-dimethylthetin chloride. Control determinations of the initial rate with dimethylthetin as methyl donor were included in every set of manometric experiments carried out.

Table 1. *Activity of various sulphonium halides as substrates of thetin–homocysteine transmethylase*

Substrates: DL-homocysteine (0·03 M), sulphonium halides (0·1 M); 0·5 ml. of enzyme preparation in side arm of flask; 0·02 M-bicarbonate medium; flasks gassed with $N_2 + CO_2$ (95:5, v/v); temp. 37·1°. Results expressed as percentages of the initial rate obtained with dimethylthetin chloride.

Compound	Initial rate
Dimethylthetin chloride	100
Ethylmethylthetin chloride	85·0
Diethylthetin chloride	0
Dimethyl-α-propiothetin bromide	51·9
Dimethyl-β-propiothetin bromide	24·9
Ethylmethyl-β-propiothetin bromide	33·0
Dimethyl-γ-butyrothetin bromide	23·6
Methionine methylsulphonium chloride	17·5*
Sulphocholine iodide	0
Trimethylsulphonium chloride	16·5
Triethylsulphonium chloride	0
Ethyldimethylsulphonium iodide	6·6
Butyldimethylsulphonium iodide	4·0

* Corrected for CO_2 retention produced by this compound.

It was not convenient to employ sulphonium salts of the same halide throughout the series of experiments. However, variation of the halide ion in several of the compounds used had no significant effect on the initial reaction rate and, further, the presence of added sodium chloride, bromide or iodide in concentrations up to 0·2 M was also without effect.

Almost all the methylsulphonium salts tested acted as substrates, although none was more effective than dimethylthetin itself. The ability of thetins to donate methyl groups decreased with increased chain length, as seen from the rates obtained with dimethyl-α-propiothetin, dimethyl-β-propiothetin and dimethyl-γ-butyrothetin. The rate for methionine methylsulphonium chloride was about 17 % of that for dimethylthetin, but this figure is approximate, as allowance had to be made for the large retention of carbon dioxide produced by this compound under the experimental conditions employed (owing to its buffering action and to the fact that it was used in relatively high concentration). An unexpected finding was the ability of trimethylsulphonium salts to act as substrates. On the other hand, sulphocholine iodide was completely ineffective.

Diethylthetin and triethylsulphonium chlorides did not give rise to formation of carbon dioxide, indicating the lack of occurrence of a transethylation reaction under these conditions. For a compound to act as a substrate, it must clearly possess a methyl group attached to the sulphonium pole, although only one methyl group need be present, as evidenced by the reaction rates for ethylmethylthetin and ethylmethyl-β-propiothetin, which are not far removed from those of the corresponding dimethyl compounds. Attempts to test esters of dimethylthetin as substrates have so far been unsuccessful. The methyl ester of dimethylthetin was synthesized for this purpose but was found to be too rapidly hydrolysed to dimethylthetin under the conditions of the assay to be of value as a potential substrate. Also, attempts by various methods to prepare the butyl ester of the thetin have led in every case to the formation of trimethylsulphonium salts in almost quantitative yield.

It was reported in an earlier paper (Maw, 1956) that, with dimethylthetin as the methyl donor, production of carbon dioxide by the thetin transmethylase preparations was not linear, but fell off slightly with time. In addition, the relationship between initial reaction rate and enzyme concentration was also non-linear, so making it impossible to assay the enzyme accurately by the manometric method.

With trimethylsulphonium salts as methyl donors, however, it has now been found that both the rate of formation of carbon dioxide and the

reaction rate/enzyme concentration relationship are strictly linear, as shown in Figs. 1 and 2. At the present time, the most suitable substrate for use in the assay of the thetin transmethylase by the manometric method would therefore appear to be trimethylsulphonium chloride, despite the relatively low reaction rates obtained with this compound. In the series of experiments described in this paper, which have employed dimethylthetin and other thetins as substrates, the problem of the non-linear reaction rate/enzyme concentration relationship obtained with these compounds has been circumvented by determining all reaction rates not only at fixed substrate concentrations, but also at the same enzyme concentration.

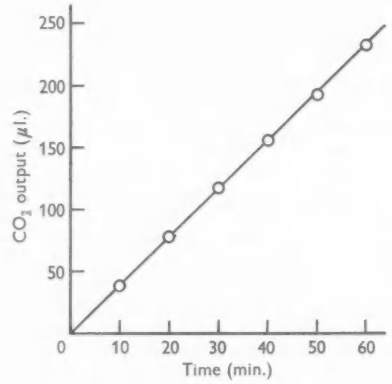

Fig. 1. Formation of carbon dioxide by a thetin–homocysteine transmethylase preparation from rat liver. Substrates: DL-homocysteine (0·03 M), trimethylsulphonium chloride (0·2 M): 0·5 ml. of enzyme preparation in side arm of flask; 0·02 M-bicarbonate medium; flasks gassed with N_2+CO_2(95:5, v/v); temp. 37·1°.

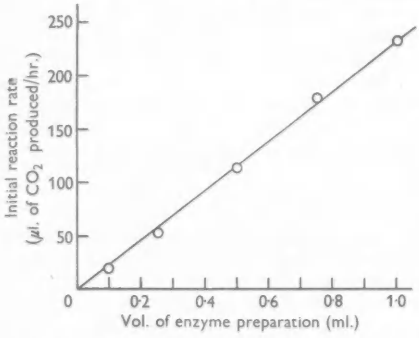

Fig. 2. Activity/concentration relationship for a thetin–homocysteine transmethylase preparation from rat liver. Substrates: DL-homocysteine (0·03 M), trimethylsulphonium chloride (0·2 M). Other details as in Fig. 1.

Specificity of the enzyme towards methyl acceptors. Several thiols have been tested in place of DL-homocysteine as methyl acceptors for the thetin transmethylase, with dimethylthetin as methyl donor. Cysteamine, 2-mercaptoethanol and sodium mercaptoacetate at 0·01 and 0·03 M were ineffective. With L-cysteine there was a small but steady production of carbon dioxide, the initial rate at 0·03 M-cysteine being equivalent to 12 % of that obtained with the same concentration of DL-homocysteine. Experiments are in progress to determine whether this formation of carbon dioxide corresponds to a methylation of cysteine taking place under these conditions.

DL-Homocystine was completely ineffective as a substrate for the enzyme. This is in accord with the current belief that the disulphide must undergo reduction to homocysteine before its methylation in the animal.

Inhibition of the transmethylase

Effects of analogues. Manometric experiments have been carried out to determine whether any of the above-mentioned sulphonium compounds and thiols which are not substrates for the transmethylase act as inhibitors of the methylation of homocysteine by dimethylthetin. The concentrations of methyl donor and acceptor were 0·1 and 0·03 M respectively. Diethylthetin and sulphocholine showed no inhibitory effects at concentrations up to 0·1 M. The nitrogen analogue of dimethylthetin, glycine betaine, was without effect at concentrations up to 0·05 M, but consistently produced inhibitions in the region of 20 % at a concentration of 0·1 M.

Of the thiols tested, cysteine at concentrations up to 0·03 M produced no effect other than a retention of carbon dioxide attributable to its buffering action. 2-Mercaptoethanol was also without effect. Sodium mercaptoacetate, however, acted as an inhibitor, and at a concentration of 0·03 M caused a reduction in initial reaction rate of 26 %. An inhibition of the thetin–homocysteine transmethylase of pigeon liver by mercaptoacetate has been reported by Sloane *et al.* (1956), who found that the effect of this compound could be overcome by the addition of excess of the substrates, suggesting that the inhibition was competitive. Sodium sulphide (0·03 M) had no inhibitory effects on the rat-liver enzyme.

Effects of metal ions. The activity of the transmethylase was markedly affected by the presence of a variety of metal ions. None of those tested caused activation, but several of the heavy-metal ions were highly inhibitory, as shown in Table 2.

These include Co^{2+}, Cu^{2+}, Zn^{2+} and Hg^{2+} ions the last having a pronounced effect at mM. Fe^{2+} ions were also inhibitory at this concentration. Ca^{2+} and Mg^{2+} ions had negligible effects, even at 0·01 M. Ag^{+} ions produced a 35% inhibition at 0·01 M. It is of interest that K^{+} ions also proved to be an inhibitor and to have an effect at concentrations up to 0·01 M comparable with that of Ag^{+} ions. The effect of K^{+} ions was detectable at 0·1 mM. In one experiment, with KCl at concentrations of 0·1 mM, 1 mM, 0·01 M and 0·1 M, the inhibitions obtained were 9, 18, 25 and 29% respectively. That the effect of potassium salts was due solely to the K^{+} ion was indicated by identical inhibitions obtained for potassium chloride, bromide and sulphate over a wide range of salt concentrations. Rb^{+} ions also showed inhibitory effects, although they were weaker than those of K^{+} ions, namely 13% inhibition at mM, and 17% at 0·01 M-RbCl. Li^{+} and Cs^{+} ions, on the other hand, showed no inhibitory effects at all the concentrations employed.

Several of the heavy-metal salts used, e.g. those of Hg^{2+} and Ag^{+}, gave precipitates when added to the incubation medium. By combining with the homocysteine or with the bicarbonate present, or with both constituents, these salts might therefore have produced an apparent inhibition by altering the buffering power of the medium or by reducing the concentration of methyl acceptor. Experiments have shown, however, that even if the addition of a heavy-metal salt at 0·01 M caused the bicarbonate concentration to be halved or the homocysteine concentration to be reduced by one-third, the maximum reduction in rate to be expected would be no more than about 12%, which is very much less than the inhibitions actually observed. Further evidence that the effect of these metal ions is primarily on the enzyme rather than on the medium or the substrates was obtained by adding the appropriate salts to the enzyme in the side arm of each Warburg flask at the beginning of the experiment. The enzyme was thus incubated with a concentrated solution of the metal salt for a preliminary period of about 20 min. before it was mixed with the substrates and the main bulk of the medium. The degree of inhibition obtained in these circumstances was the same as that observed when the salts were added initially to the medium and substrates. This finding may also be taken to indicate that the substrates afford the enzyme no protection against the effects of heavy-metal ions.

Effects of some other enzyme inhibitors. Certain of the more commonly-used enzyme inhibitors were tested for their effects on the transmethylase, with dimethylthetin acting as the methyl donor. Sodium azide, sodium cyanide and sodium fluoride produced no inhibitory effects at concentrations up to 0·01 M. Ethylenediaminetetra-acetic acid (EDTA) was also without effect at concentrations up to 2 mM. With the carbonyl reagents hydroxylamine and phenylhydrazine no effects were observed at a concentration of mM, while at 0·01 M the two compounds produced inhibitions of 32 and 18% respectively. Semicarbazide caused no significant inhibition at either concentration.

In testing the effect of iodoacetic acid on the enzyme, allowance had to be made for the rapid non-enzymic reaction which takes place between the compound and the thiol group of homocysteine. This reaction reduces the concentration of methyl acceptor, but at the same time liberates protons and so results in the formation of carbon dioxide from the bicarbonate buffer. The iodoacetate was consequently added initially to the enzyme in the side arm of the Warburg flask, so that the enzyme was in contact with the inhibitor for a period of at

Table 2. *Effect of various metal ions on the activity of thetin–homocysteine transmethylase*

Substrates: DL-homocysteine (0·03 M), dimethylthetin chloride (0·1 M); concentrations of salts added to main compartment of flask, 0·001 M and 0·01 M; other details as for Table 1.

Salt	Percentage inhibition produced by 0·001 M-salt	Percentage inhibition produced by 0·01 M-salt
LiCl	0	0
KCl	24·3	28·8
RbCl	12·6	17·3
CsCl	0	0
$CuSO_4$	10·2	100
$AgNO_3$	17·2	34·8
$CaCl_2$	0	0
$MgCl_2$	0	0
$ZnCl_2$	16·1	100
$HgCl_2$	63·3	100
$MnCl_2$	0	24·8
$FeSO_4$	34·4	48·4
$CoCl_2$	9·8	85·8

Table 3. *Effect of iodoacetic acid on the activity of thetin–homocysteine transmethylase*

Substrates: DL-homocysteine (0·03 M), dimethylthetin chloride (0·1 M); other details as for Table 1.

Concn. of iodoacetic acid (mM)	Enzymic reaction rate	Non-enzymic reaction rate	Expected combined rate	Observed rate	Percentage inhibition
0·1	94·7	<1	95	98·4	—
1	94·7	4·7	99·4	102·7	—
10	94·7	80·7	175·4	147·5	16

least 20 min. before mixing with the substrates took place. The initial reaction rate obtained under these conditions is the sum of the rates of the simultaneous non-enzymic and enzymic reactions. If iodoacetate were to produce any inhibitory effect on enzyme activity, this observed initial rate should therefore be less than the sum of the non-enzymic reaction rate and the rate of the enzymic reaction in the absence of iodoacetate. By using three different iodoacetate concentrations, as shown in Table 3, only at the highest inhibitor concentration (0·01 M) was the observed rate less than the sum of the two component rates.

Although these results are not conclusive, they do suggest that the activity of the transmethylase is relatively insensitive to the action of this inhibitor.

DISCUSSION

A comparison may be made of the results obtained in the present series of experiments with previous observations on the metabolism of sulphonium compounds in the intact rat. Dimethylthetin and dimethyl-β-propiothetin are efficient methyl donors *in vivo*, judged by their ability to support the growth of rats on methyl-deficient diets (Maw & du Vigneaud, 1948*a*, *b*). Both compounds also undergo extensive oxidation to inorganic sulphate in the rat (Maw, 1953). This is explained by their ability to act as substrates for the thetin–homocysteine transmethylase, for there is evidence that the initial step in their oxidation is a demethylation (Maw, 1954). The variable activity of ethylmethylthetin *in vivo* may be contrasted with its effectiveness as a methyl donor in enzyme experiments. This difference in behaviour may be due to factors which are operative *in vivo*, e.g. the toxicity of the demethylated product of the thetin, ethylthioacetic acid.

The sulphonium salt, sulphocholine chloride, has been found unable to serve as an efficient source of methyl groups *in vivo* (Maw & du Vigneaud, 1948*c*). On the other hand, in tissue preparations under aerobic conditions this compound has been shown to be involved in the methylation of homocysteine (Dubnoff, 1949) and, further, in the intact rat it is to some extent oxidized to sulphate (Maw, 1953). The ineffectiveness of sulphocholine as a substrate for the thetin transmethylase argues against its participation as a direct methyl donor, but is in accord with the belief that this compound undergoes a preliminary oxidation to dimethylthetin as a first step in its metabolism (Dubnoff, 1949; Maw, 1953).

The finding that methionine methylsulphonium chloride is a substrate for the transmethylase accounts for its ability to act *in vivo* in place of methionine. In view of its wide occurrence in vegetables (Challenger & Hayward, 1954; McRorie *et al.* 1954), this compound must therefore be regarded as a potential, if minor, source of dietary methyl groups for the animal. Durell *et al.* (1957) have recently drawn attention to the high concentration of thetin–homocysteine transmethylase in horse liver, and have found that *S*-adenosylmethionine does not act as a substrate for the enzyme. This raises the possibility that methionine methylsulphonium salts, and perhaps also the simpler thetins, may be of more importance in animal metabolism than was originally supposed.

No evidence has yet been obtained to suggest that the transmethylase possesses a prosthetic group. The enzyme resists prolonged dialysis, is not markedly affected by carbonyl reagents and is unaffected by the presence of EDTA, and no activation has been observed with a variety of metal ions. Its mode of action has also not been established. Two possible mechanisms have been envisaged and are proposed here. The first is that one of the active centres of the enzyme may consist of a tertiary nitrogen atom or a bivalent sulphur atom which acts as the initial acceptor of methyl groups from methylsulphonium compounds by participating in a group transfer reaction. This is shown below, assuming the active centre ($N \equiv E$) to be tertiary nitrogen:

$$R \cdot \overset{+}{S}(CH_3)_2 + N \equiv E \rightarrow R \cdot S \cdot CH_3 + CH_3 \cdot \overset{+}{N} \equiv E$$

The quaternary-ammonium (or sulphonium) form of the enzyme would then transfer the methyl group to homocysteine as follows:

$$CH_3 \cdot \overset{+}{N} \equiv E + HS \cdot R' \rightarrow N \equiv E + CH_3 \cdot \overset{+}{S}H \cdot R'$$
$$(\rightarrow CH_3 \cdot S \cdot R' + H^+)$$

The numerous tissue constituents containing tertiary nitrogen which might be capable of acting as initial acceptors include histidine, nicotinamide and other heterocyclic structures, as well as dimethylethanolamine and related compounds.

An alternative mechanism involves the preliminary combination of the enzyme with homocysteine. This is a more likely possibility in view of the high specificity of the enzyme for this substrate. The role of the enzyme would be to bind the homocysteine, presumably through its amino and carboxyl groups, and by bringing the thiol group into close proximity with some basic group to induce liberation of a proton. This would be followed by a thiolytic attack of the mercaptide ion thus formed on the methyl carbon atom of a methyl-donor substrate. The two schemes differ in that the first requires the combination of enzyme with methyl donor as the preliminary step, whereas the second requires an initial combination between enzyme and methyl acceptor. These two possibilities correspond respectively to the double and

single displacement mechanisms for enzymic substitution reactions recently discussed by Koshland (1954).

SUMMARY

1. The ability of a number of methylsulphonium and ethylsulphonium salts related to dimethylthetin to act as substrates for partially purified preparations of thetin–homocysteine transmethylase from rat liver has been examined. Dimethylthetin is the most effective methyl donor so far investigated.

2. Trimethylsulphonium chloride acts as a substrate for the enzyme, and gives rise to a linear reaction rate and a linear enzyme activity/concentration relationship. This compound has therefore been considered as a suitable substrate for the manometric assay of the transmethylase.

3. Homocysteine was the only effective methyl acceptor among the thiols tested, although some evidence was obtained to suggest that L-cysteine might be acting as a substrate.

4. None of the metal ions which were tested activated the enzyme; several heavy-metal ions and potassium ions were inhibitory. Several other commonly-used inhibitors had no effect on the enzyme.

5. Possible mechanisms for the participation of the enzyme in the transmethylation reaction have been proposed.

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Further Observations on the Oxidation of Di-iodotyrosine Derivatives

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It has already been shown by one of us (Pitt-Rivers, 1948) that the conversion of di-iodotyrosine into thyroxine *in vitro* is enhanced when both the amino and carboxyl groups of di-iodotyrosine are blocked; the first compound of this nature to be studied was *N*-acetyl-DL-di-iodotyrosyl-L-glutamic acid, which gave rise to the corresponding thyroxine derivative in yields up to 35%, calculated on amount of di-iodotyrosine derivative lost. In the present paper, the formation *in vitro* of several new thyroxine derivatives is described. At the same time, the side chain lost from one di-iodotyrosine molecule during the coupling reaction has been identified.

EXPERIMENTAL

The acetyl-DL-di-iodotyrosyl-amino acid derivatives were all formed from 4-(3:5-di-iodo-4-acetoxybenzyl)-2-methyloxazolone and the required amino acid, as in the synthesis of *N*-acetyl-DL-di-iodotyrosylglutamic acid (Pitt-Rivers, 1948) and will not, except for *N*-acetyl-DL-di-iodotyrosyl-ε-*N*-(α-*N*-acetyl)-lysine, be described in detail; the aerobic incubation of this compound, which gave the highest yields of the thyroxine derivative, was extensively studied and led to the recognition of the C_3 residue split off from the di-iodotyrosine molecule.

*N-Acetyl-*DL*-di-iodotyrosyl-*ε*-N-(*α*-N-acetyl)-*L*-lysine*

4-(3:5-Di-iodo-4-acetoxybenzyl)-2-methyloxazolone (20 g.) was dissolved in dry acetone (170 ml.) and chilled in an ice bath. α-*N*-Acetyl-lysine (Neuberger & Sanger, 1943) (6 g.) dissolved in N-NaOH (32 ml.) was added and the reaction was allowed to proceed for 14 hr. Most of the acetone was then removed under diminished pressure, the solution was diluted with water, and acidified with HCl. After chilling, the product was collected, washed with water and crystallized from 50% (v/v) ethanol. The *compound* crystallized in thin leaflets, m.p. 170–171°; the yield was 88% of the theoretical (Found: N, 6·4; I, 39·4. $C_{19}H_{25}O_6N_3I_2$ requires N, 6·5; I, 39·4%).

Incubation of N*-acetyldi-iodotyrosyl-*ε*-*N*-(*α*-*N*-acetyl)-*L*-lysine*

The above compound (12·9 g., 0·2 mole) was suspended in 100 ml. of 0·2 M-borate buffer (pH 7·5) and dissolved by addition of 35 ml. of N-NaOH. Saturated Na_2SO_4 solution (35 ml.) was added, since it was found to produce filtrable crystals of the sodium thyroxine derivative. The solution was kept at 38° for 10–11 days in an open Erlenmeyer flask (700 ml.) without shaking, after which a heavy crystalline deposit of the sodium salt of *N*-acetyl-DL-thyroxyl-ε-*N*-(α-*N*-acetyl)-L-lysine had separated. This was filtered off, washed with borate buffer and decomposed by acidification of its alkaline solution. The *thyroxine derivative* was recrystallized from 70% (v/v) ethanol, and separated in fine needles, which had an indeterminate m.p. with final decomposition at 200° (Found: C, 30·1; H, 3·0; N, 4·3; I, 51·0. $C_{25}H_{27}O_7N_3I_4$ requires C, 30·3; H, 2·7; N, 4·25; I, 51·4%).

As shown in Table 1, the yield of the thyroxine derivative varied from 40 to 50%, calculated on the recovered di-iodotyrosine derivative; the latter after recrystallization from 50% ethanol plus charcoal could be used for further incubation experiments.

Compounds giving rise to thyroxine derivatives

In Table 1 are shown the di-iodotyrosine derivatives which give rise to thyroxine derivatives and the net yields obtained after 10–11 days' aerobic incubation at pH 7·5 and 38°, in open Erlenmeyer flasks (700 ml.).

As is seen, the yields were variable and in most instances the reaction mixtures at the end of the experiment had a brownish colour, indicating decomposition of the di-iodotyrosine derivative. This was not so with the lysine peptide, where the reaction mixture remained a pale-straw colour throughout the incubation. For this reason it was chosen for the investigation of other products of the reaction.

Other products formed during the incubation of N*-acetyldi-iodotyrosyl-*ε*-*N*-(*α*-*N*-acetyl)-lysine*

In the initial incubation experiments, a thorough search was made in the reaction mixture for serine, alanine and oxalic acid. In no case could any of these be detected, although it was shown that recovery of all these compounds when added to the reaction mixture was good.

Acetamide. A little free NH_3 was shown to be present in the reaction mixture, and it was therefore suspected that acetamide might be present, resulting from oxidative deamination of *N*-acetyldi-iodotyrosine. Ammonia and acetamide were determined according to the method of Pucher, Vickery & Leavenworth (1935). In two experiments, where the amount of thyroxine derivative obtained was 0·51 and 0·57 m-mole, the NH_3 plus acetamide was found to be 0·52 and 0·585 m-mole respectively. The free NH_3 represented less than 10% of the NH_3 lost. It therefore appears that in these experiments the formation of thyroxine is accompanied by an equivalent loss of NH_3 (as acetamide) from the side chain of di-iodotyrosine. This led to a search for a carbonyl derivative which might be quantitatively isolated.

The carbonyl derivative from the di-iodotyrosine side chain. Early attempts to isolate the carbonyl derivative with 2:4-dinitrophenylhydrazine were unsatisfactory and *p*-bromophenylhydrazine was substituted. After removal of unchanged di-iodotyrosine peptide by acidification, the reaction mixture was treated with an amount of *p*-bromophenylhydrazine molecularly equivalent to the amount of thyroxine peptide obtained. After a short time, a pale-yellow crystalline precipitate appeared; separation was complete after 3 days at 4°; further addition of *p*-bromophenylhyrazine did not increase the precipitate. Chromatographic analysis of the yellow material in butanol–dioxan–2N-NH_3 (4:1:5, by vol.) showed that the major component was colourless, and absorbed in u.v. light (substance *A*). There was also present a bright-yellow component which fluoresced in u.v. light (substance *F*). These two compounds were not satisfactorily separated by crystallization, but *A* was soluble in hot water whereas *F* was not. The crude material was therefore extracted with water in a Soxhlet apparatus and the crystalline substance *A* so obtained was recrystallized from 30% (v/v) methanol, from which it separated in glistening plates, m.p. 160–162° (Found: C, 46·0; H, 5·0; N, 12·6; Br, 18·0%).

This analysis agrees with that required for the p-*bromophenylhydrazone* of ε-*N*-hydroxypyruvoyl-α-*N*-acetyl-lysine, which has C, 46·0; H, 5·0; N, 12·65; Br, 18·1%. The amounts of the bromophenylhydrazone obtained after incubation varied between 80 and 90% of the amounts

Table 1. *Di-iodotyrosine derivatives giving rise to thyroxine derivatives during aerobic incubation*

Di-iodotyrosine derivative	Thyroxine derivative formed	Net yield of thyroxine derivative (%)
N-Formyl-L-di-iodotyrosine	*N*-Formyl-L-thyroxine	20
N-Propionyl-L-di-iodotyrosine	*N*-Propionyl-L-thyroxine	20
N-Acetylglycyl-L-di-iodotyrosine	*N*-Acetylglycyl-L-thyroxine	30
N-Acetyl-DL-di-iodotyrosylglycine	*N*-Acetyl-DL-thyroxylglycine	30
N-Acetyl-DL-di-iodotyrosyl-β-alanine	*N*-Acetyl-DL-thyroxyl-β-alanine	10
N-Acetyl-DL-di-iodotyrosyl-L-valine	*N*-Acetyl-DL-thyroxyl-L-valine	10
N-Acetyl-DL-di-iodotyrosyl-L-proline	*N*-Acetyl-DL-thyroxyl-L-proline	15
N-Acetyl-DL-di-iodotyrosyl-ε-*N*-(α-*N*-acetyl)-L-lysine	*N*-Acetyl-DL-thyroxyl-ε-*N*-(α-*N*-acetyl)-L-lysine	40–50
4-Hydroxy-3:5-di-iodophenylacetic acid*	Tetraiodothyroacetic acid	15–20

* Although not a derivative of di-iodotyrosine, this compound has been included since it gives considerable yields of the thyroxine analogue.

expected from the yields of the thyroxine derivative obtained.

Reduction of substance A. The purified bromophenylhydrazone, *A*, was suspended in water and shaken with sodium amalgam at room temperature (cf. Sprinson & Chargaff, 1946). When hydrogen ceased to be evolved the aqueous solution was neutralized and then further acidified to 2N with HCl. After boiling under reflux for 2½ hr., the solution was concentrated and chromatographed on Whatman No. 3 paper in *m*-cresol–water in an atmosphere of NH_3. This system has been shown to separate glycine, alanine, serine and lysine. The last-named amino acid, after 4 days' descending chromatography, is washed off the paper. The R_F of the reduction product of *A*, after removal of lysine, is the same as that of serine; no alanine was found. It was therefore concluded that the whole of the di-iodotyrosine side chain had been converted into hydroxypyruvic acid.

Purification of substance F. The water-insoluble yellow material left after aqueous extraction of *A* was crystallized from ethanol, from which it separated in fine yellow needles having m.p. 225–227° (decomp.) (Found: C, 45·6; H, 4·6; Br, 26·0%). This analysis agrees with that required for the p-*bromophenylosazone* of ε-*N*-hydroxypyruvoyl-α-*N*-acetyllysine, which has C, 45·1; H, 4·3; Br, 26·2%.

The osazone was only formed in very small amounts, and was considered to be a by-product of the reaction.

Incubation of N-*acetyl*-L-*di-iodotyrosine*

This compound has been shown (Pitt-Rivers, 1948) to give rise to *N*-acetyl-L-thyroxine during aerobic incubation. Repeated attempts to characterize the side chain which is split off during the reaction have been made, by using *p*-nitrophenylhydrazine, 2:4-dinitrophenylhydrazine and *p*-bromophenylhydrazine for the isolation of the carbonyl derivative, but no pure compounds could be isolated.

Identification of the side chain after incubation of 3:5-di-iodo-4-hydroxyphenylacetic acid

This compound was incubated in borate buffer for 10 days at 37°, and, after removal of tetraiodothyroacetic acid and unchanged starting material, the solution was treated with 2:4-dinitrophenylhydrazine. The yellow precipitate so obtained was shown by chromatographic analysis in butanol saturated with 3% aqueous NH_3 to have the same R_F values as those of the dinitrophenylhydrazones of glyoxylic acid (probably obtained from a mixture of the *cis* and *trans* forms of the acid).

Di-iodotyrosine derivatives and related compounds which do not give rise to the corresponding thyroxine analogue

The following compounds gave no thyroxine derivative after aerobic incubation at pH 7·5 for 10–14 days: DL-di-iodotyrosyl-L-di-iodotyrosine, *N*-acetyldi-iodotyrosyldi-iodotyrosine, benzoyl-L-di-iodotyrosine, phenacetyl-L-di-iodotyrosine and 4-hydroxy-3:5-di-iodophenylbenzoic acid.

DISCUSSION

From these findings it appears that aerobic incubation of acetyldi-iodotyrosine peptides gives rise to the following products: the corresponding thyroxine derivative, acetamide and hydroxypyruvoylpeptide; these last are both present in amounts approximately equivalent to the amount of thyroxine found. It has already been suggested (Pitt-Rivers, 1948) that oxidation on the β-carbon atom of the di-iodotyrosine side chain probably occurs during oxidation by hydrogen peroxide since 3:5-di-iodo-*p*-hydroxybenzaldehyde was

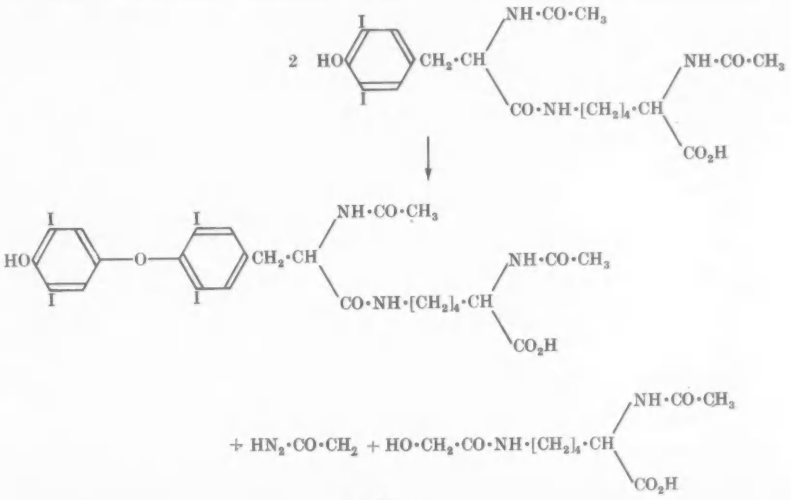

Fig. 1

obtained as a by-product. However, in the milder conditions used in the present experiments the further breakdown of the side chain does not take place, and the reaction may be shown to follow quantitatively the scheme shown in Fig. 1. It must again be emphasized that, under the conditions of these incubations, the thyroxine derivative, acetamide and the hydroxypyruvoyl-lysine derivative are the only compounds obtained. Thus we have been unable to confirm the claim by Johnson & Tewkesbury (1942) that pyruvic acid was formed during alkaline incubation of di-iodotyrosine; nor have we been able to demonstrate the presence of serine, which Sela (1956) found among the products obtained from the alkaline incubation of iodinated polytyrosine.

The fact that the hydroxypyruvic acid derivative can be isolated at all is probably due to the blocking of its carboxyl group. Attempts to form a *p*-bromophenylhydrazone from solutions of hydroxypyruvic acid prepared according to Sprinson & Chargaff (1946) led to gummy decomposition products. The latter authors have also demonstrated the instability of hydroxypyruvic acid in alkaline solution. This may account for the failure to detect it when the carboxyl group of di-iodotyrosine is not blocked, i.e. after the incubation of *N*-acetyldi-iodotyrosine.

It seems worth commenting on the fact that a di-iodo-*p*-hydroxyphenyl derivative with a C_2 side chain can also give rise to a tetraiodiphenyl ether. The formation of tetraiodothyroacetic acid from 4-hydroxy-3:5-di-iodophenylacetic acid occurred in yields as good as those of acylated thyroxines obtained from the acylated di-iodotyrosines; it may be that an essential feature during the formation of tetraiodiphenyl ether is oxidation of the side-chain carbon atom adjacent to the ring (Fig. 2). Certainly in the absence of this methylene group no condensation occurs, i.e. as with 4-hydroxy-3:5-di-iodobenzoic acid.

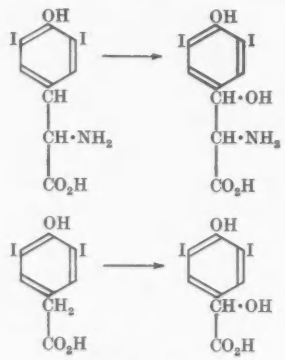

Fig. 2

A final comment may be made about other di-iodotyrosine derivatives which do not give a thyroxine derivative during mild alkaline oxidation, namely benzyl- and phenacetyl-di-iodotyrosine, di-iodotyrosyldi-iodotyrosine, *N*-acetyldi-iodotyrosyldi-iodotyrosine and 4-hydroxy-3:5-di-iodophenylpyruvic acid. The marked instability of the last compound in alkaline solution probably leads to its complete breakdown, as evidenced by the green colour rapidly obtained during incubation. With the other compounds steric hindrance may be the predominant cause for the failure to couple; in each, the amino group of di-iodotyrosine is attached to a group containing another phenyl residue. Table 1 shows that only di-iodotyrosine derivatives acylated with completely aliphatic groups will give rise to thyroxine.

SUMMARY

1. The formation of thyroxine peptides from di-iodotyrosine peptides has been further investigated.

2. *N*-Acetyldi-iodotyrosyl-ε-*N*-(α-acetyl)-lysine has been shown to give rise to high yields of the corresponding thyroxine derivative during aerobic incubation at pH 7·5.

3. The side chain lost during incubation of this peptide has been characterized through its *p*-bromophenylhydrazone as ε-*N*-hydroxypyruvoyl-α-*N*-acetyl-lysine.

4. The side chain lost from *N*-acetyl-L-di-iodotyrosine during oxidative coupling has not been satisfactorily characterized. It has not been possible to demonstrate the presence of pyruvic acid.

5. The side chain lost during the formation of tetraiodothyroacetic acid from 4-hydroxy-3:5-di-iodophenylacetic acid has been identified as glyoxylic acid.

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